Manuscript Details

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Title	Mutant p53 induces SIRT3/MnSOD axis to moderate ROS production in melanoma cells
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Abstract

The TP53 tumor suppressor gene is the most frequently altered gene in tumors and mutant p53 isoforms can acquire oncogenic properties referred to as gain-of-function (GOF). In this study, we used wild-type (A375) and mutant p53 (MeWo) melanoma cell lines to assess the regulation of the mitochondrial antioxidant manganese superoxide dismutase (MnSOD) by mutant p53. The effects of mutant p53 were evaluated by qPCR, immunoblotting, enzyme activity assay, cell proliferation assay, reactive oxygen species (ROS) assay after cellular transfection. We demonstrate that mutant p53 induces MnSOD expression, which is recovered by the ROS scavenger N-acetyl-L-cysteine. This suggests MnSOD induction as a defense mechanism of melanoma cells to counterbalance the pro-oxidant conditions induced by mutant p53. We also demonstrate that mutant p53 induces the expression of Sirtuin3 (SIRT3), a major mitochondrial NAD+-dependent deacetylase, stimulating MnSOD deacetylation and enzymatic activity. Indeed, the restoration of SIRT3 reverses MnSOD activity decrease by mutant p53 knock-down. Finally, MnSOD knock-down further enhances mutant p53-mediated ROS increase, contracting mutp53-dependent cell hyperproliferation. This indicates that SIRT3 and MnSOD act to maintain ROS levels controlled to promote cell proliferation and survival, providing new therapeutic opportunities to be further considered for clinical studies in cancer patients bearing mutant TP53 gene.

Keywords	ROS; mutant p53; gain-of-function; cancer; SIRT3; MnSOD
Taxonomy	Molecular Biology, Biochemistry

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UNIVERSITY OF VERONA
DEPARTMENT OF NEUROSCIENCES, BIOMEDICINE AND MOVEMENT
SECTION OF BIOLOGICAL CHEMISTRY

Archives of Biochemistry and Biophysics Verona, November 11th 2019

Editorial Office

Dear Dr. Henry Jay Forman

We wish to thank you very much for your efforts in handling our manuscript (Ref: YABBI_2019_482; Title: "Mutant p53 induces SIRT3/MnSOD axis to moderate ROS production in melanoma cells") and for giving us the opportunity to revise and improve it. We greatly appreciated your thoughtful and those from the reviewers. We revised the manuscript accordingly.

Dear Dr. Donadelli,
Thank you for submitting your manuscript to Archives of Biochemistry and Biophysics. Your manuscript has been examined by two reviewers, who have concluded that the work may be appropriate for publication in Archives of Biochemistry and Biophysics. However, as indicated in the enclosed comments, the reviewers have concluded significant revisions and additional data are required. A revised manuscript will be returned to reviewers for their comments prior any decision regarding publication. The revision is due 60 days from the date of this letter.
When resubmitting revised your manuscript, please respond to all of the reviewers' comments in a separate document that describes each change, and provides suitable rebuttals for any comments not addressed by a change in the manuscript.

We have performed additional experiments to address the concerns regarding the comments of the Reviewers. In particular, we strengthened some controls using different gene knock-down tools and we also improved the results on the outcome of the cells on the mechanisms identified in our manuscript. We have also modified the text on the basis of Reviewers' criticisms. We hope that all Reviewers' requirements have been satisfied and that this revised version of our manuscript is acceptable for publication in ABB.

Yours sincerely,

Massimo Donadelli

Archives of Biochemistry and Biophysics Verona, November 11th 2019

Editorial Office

Dear Dr. Henry Jay Forman,

Please find below the detailed, itemized list of our responses to the Reviewers' suggestions/comments and the changes we have made in the revised version of the manuscript.

Comments from the editors and reviewers:
-Reviewer 1

- In the present work the authors have identified a new mechanism by which p53mutation is able to regulate the oxidative stress levels in melanoma cells via the activation of SIRT3 which can deacetylate MnSOD and increase the antioxidant cellular defense. The authors were able to support this theory by the use of 2 different kind of melanoma cells (A375 and MeWo) where specific p53 mutant were inserted. In addition SIRT3 expression was manipulated as proof of concept of its involvement in MnSOD activation/expression.

The work is well conducted and the data are solid

Comments:
- In the methods the authors described the measurement of H2O2 while in the results they mention the generic term "ROS". Please use H2O2 as ROS is too broad beside the fact that H2O2 was the one detected.
Response: We thank the Reviewer for this suggestion. Accordingly, we have substituted ROS with H_2O_2 when we referred to the specific result described in the present manuscript (for example in Material and Methods; Results; Legends; Figures). When the term ROS was used to describe a general concept we maintained the term ROS (Introduction, Discussion).

- Do the authors think that this effect can be translated also to the cytosolic SOD? please discuss this aspect
Response: This is an interesting point that we could plan to develop in future projects. However, our preliminary data indicate that mutant p53 knock-down fails to significantly modulate cytosolic Cu/ZnSOD expression in cancer cells, suggesting the involvement of MnSOD rather than Cu/ZnSOD as an antioxidant defense system. Thus we developed the present study specifically on MnSOD. However, to address this point future investigations on the regulation of the Cu/ZnSOD activity by mutant p53 will be needed.

- in Fig. 6 should be eliminated the low-left gray box, in this study NOX, etc were not measured and not being a review article the authors should limit the graphical abstract to what showed in the study
Response: We agree with the Reviewer. Of course, to provide to the readers a comprehensive discussion we have maintained the information on the regulation of ROS-related genes by mutant p53 in the Discussion section of the manuscript, but we have deleted the gray box in Figure 6.

- Have the authors tried to add exogenous H2O2 and evaluate the levels of SIRT3 and MnSOD? Do the authors think that other defensive enzyme (GPX, CAT, etc) can be further players in the suggested pathway?
Response: We thank the Reviewer for his/her suggestion, which improved our manuscript. In the revised version of our manuscript we have inserted new qPCR data of MnSOD and SIRT3 mRNA

expression after cell treatment with exogenous H_2O_2 (Figures 2D and 3B). As discussed in the text, these new data further support the concept by which cancer cells bearing mutant *TP53* gene can induce the MnSOD/SIRT3 axis as a cytoprotective mechanism triggered by ROS. On the other side, we did not observe MnSOD/SIRT3 regulation in WT-p53 cells treated with exogenous H_2O_2, suggesting that the basal endogenous antioxidant p53-target genes can efficiently counterbalance the exogenous addition of H_2O_2. Concerning other defense enzymes as GPX or CAT, we cannot exclude their involvement as a cytoprotective mechanism induced by mutp53-dependent induction of ROS. However, our preliminary data indicate that mutant p53 fails to significantly modulate them, suggesting that the mechanism identified in our manuscript may primarily involve mitochondrial enzymes.

-Reviewer 2

In the present report the authors present evidence that mutant p53^{E258K} found in melanoma cells controls in a balanced manner ROS production in order to favor cancer cell progression. Particularly the authors unravel a network into which mutant p53 1) upregulates MnSOD expression at transcriptional level, 2) generates ROS that sustain MnSOD expression and 3) increases SIRT3 expression that in turn promotes MnSOD activity.

This is a very interesting story that will attract the attention of future readers. However, before reaching publication level, some issues require attention.

Point 1. The authors perform a series of genetic manipulations (eg silencing of p53, SIRT3 and MnSOD) to confirm the validity of the investigated network. There is a lack of experiments showing the outcome on the cells, like cell cycle status, induction of apoptosis or senescence. The validity of the defined network would increase if it has an impact at cellular level.

Response: We thank the Reviewer for his/her positive comments on our work. Concerning the outcome on the cells of the mechanism described in our manuscript we have added new data in Figure 5D indicating that the inhibition of apoptosis by mutant p53 was recovered in MnSOD knocking-down conditions. These results, together with data reported in Figure 5C (ROS production) and 5E (cell proliferation), support the role of MnSOD induction as a ROS detoxifying mechanism, which can support the oncogenic (antiapoptotic and hyperproliferative) effects of mutant p53.

Point 2. If the antibodies used are appropriate for immunohistochemistry an in vivo examination in representative melanoma clinical samples showing co-expression of MnSOD with SIRT3 would tremendously boost this work.

Response: We basically agree with the Reviewer, but unfortunately we don't have melanoma clinical samples that can be used for this purpose.

Point 3. I noticed in Figure 3 that silencing of p53 was not so effective in MeWo cells. Moreover, in materials and methods it appears that only one siRNA/target was used. To avoid off-targets effects three independent siRNA/target should be used.

Response: we thank the Reviewer for his/her notification. We have specified in the materials and methods the usage of a smart pool of three oligonucleotides to silence p53. However, to further confirm that the regulation of MnSOD and SIRT3 expression is dependent on mutp53 and that it is not an off-target effect, we transduced MeWo cells with lentiviruses to inhibit mutp53 expression and analyzed mRNA expression levels of MnSOD and SIRT3. The data are in line with those

previously obtained with liposome-mediated transfection and are reported in the new Supplementary Figure 1.

Point 4. Oxidative stress can activate the DNA damage response (DDR) network, due to damage at DNA, and ARF, the second major anti-tumor barrier (PMID 22292438). Given the balanced co-operation between DDR and ARF to prevent tumor progression (PMID 23851489), a comment should be added on how the network proposed by the authors fits overall in cancer progression.

Response: We thank the Reviewer for this interesting comment, which could be a future development of our work to be deeply investigated. In the revised version of our manuscript, we have improved the Discussion providing additional information on the interplay between ROS, ARF and DDR. As requested, we have commented how the mechanism proposed in our manuscript can fit overall in cancer progression. Three additional references (n. 37, 38 and 39) have been inserted.

Mutant p53 induces SIRT3/MnSOD axis to moderate ROS production in melanoma cells

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Running title: Mutant p53 induces MnSOD

Keywords: ROS; mutant p53; gain-of-function; cancer; SIRT3; MnSOD; melanoma

Abstract

The *TP53* tumor suppressor gene is the most frequently altered gene in tumors and mutant p53 isoforms can acquire oncogenic properties referred to as gain-of-function (GOF). In this study, we used wild-type (A375) and mutant p53 (MeWo) melanoma cell lines to assess the regulation of the mitochondrial antioxidant manganese superoxide dismutase (MnSOD) by mutant p53. The effects of mutant p53 were evaluated by qPCR, immunoblotting, enzyme activity assay, cell proliferation assay, reactive oxygen species (ROS) assay after cellular transfection. We demonstrate that mutant p53 induces MnSOD expression, which is recovered by the ROS scavenger N-acetyl-L-cysteine. This suggests MnSOD induction as a defense mechanism of melanoma cells to counterbalance the pro-oxidant conditions induced by mutant p53. We also demonstrate that mutant p53 induces the expression of Sirtuin3 (SIRT3), a major mitochondrial NAD^+-dependent deacetylase, stimulating MnSOD deacetylation and enzymatic activity. Indeed, the restoration of SIRT3 reverses MnSOD activity decrease by mutant p53 knock-down. Finally, MnSOD knock-down further enhances mutant p53-mediated ROS increase, contracting mutp53-dependent cell hyperproliferation. This indicates that SIRT3 and MnSOD act to maintain ROS levels controlled to promote cell proliferation and survival, providing new therapeutic opportunities to be further considered for clinical studies in cancer patients bearing mutant *TP53* gene.

1. Introduction

Cutaneous melanoma is one of the most aggressive and lethal types of skin cancer that has its origins in melanocytes, especially among the white population. Its incidence is expected to grow over the next few decades due to the increasing trends in sun exposure [1]. UV radiation triggers reactive oxygen species (ROS) production, which leads to oxidative damage that may induce carcinogenesis [2]. Melanoma progression depends on many factors, especially the accumulation of genetic mutations that promote dissemination to other organs allowing cell survival to metastatic sites, in particular leading brain secondary tumors [3]. The tumor suppressor p53 can be considered the main checkpoint system of the cells, protecting them from oxidative stress via the induction of a number of antioxidant genes [4,5]. It is also a key regulator of genome integrity and cellular homeostasis through an intricate network of p53-dependent pathways, resulting in cell-cycle arrest, damage repair, senescence, apoptosis or modulation of energy metabolism [6]. However, mutations in the *TP53* gene can occur in over 50% of the human cancers and in 35% of sporadic cases of skin cancer [7]. Most of them are missense mutations that result in the expression of mutant isoforms of the p53 protein, which can acquire new biological properties referred to as gain-of-function (GOF) [8]. In addition to the loss of the tumor suppressor function of wild-type p53, GOF mutant p53 proteins contribute to the maintenance and stimulation of cancer growth through the acquisition of various oncogenic functions [9], compromising the response to anticancer treatments [10]. Different models have been proposed to explain the GOF activities of mutant p53, including binding and inactivation of the p53 family members p63 and p73 [11], modulation of the activity of a number of transcription factors, or inactivation of DNA damage molecular sensors [12,13]. It is emerging that mutant p53 proteins, contrarily to their wild-type p53 counterpart, fail to exert antioxidant properties rather sustaining a controlled increase of intracellular ROS, which favors cancer progression. In this study, we have investigated a novel survival mechanism of cancer cells induced by mutant p53, which partially counterbalances the mutant p53-dependent ROS production. This oncogenic mechanism may allow cancer cells to moderate the level of ROS increased by mutant

p53 itself, enabling them to survive even in a highly stressful oxidative environment. Our data reveal for the first time that mutant p53 can increase the expression of the key antioxidant detoxifying enzyme manganese superoxide dismutase (MnSOD) and its activity by SIRT3-mediated deacetylation in melanoma cells, contributing to temper the level of ROS and preventing their cytotoxic effects.

2. Material and Methods

2.1 Reagents

Dulbecco's modified Eagle's medium (DMEM) high glucose was obtained from Gibco (Paisley, UK). The siRNA targeting p53 (sc-29435), the siRNA targeting SIRT3 (sc-61555), the siRNA targeting MnSOD (sc-41655), and the non-targeting siRNA (sc-37007) were purchased at Santa Cruz Biotechnology (CA, USA). SIRT3 expression vector (SC127342) and pCMV6-AC control vector (PS100020) were purchased from Origene (Rockville, MD, USA). Routine chemicals were obtained from Sigma-Aldrich (Barcelona, Spain), Panreac (Barcelona, Spain) and Bio-Rad Laboratories (Hercules, CA, USA).

2.2 Cell culture and liposome-mediated transient cell transfection

A375 (WT-p53) and MeWo (mutp53^{E258K}) melanoma cell lines were used for all experiments. Cells were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotics penicillin and streptomycin and maintained in a humidified atmosphere of 5% CO_2 and 37°C. Cells were routinely tested to confirm lack of mycoplasma infection.

For siRNA transfection, 4x10^5 cells were seeded in 6-well plates, and 8x10^3 were seeded in 96-well plates. The next day, cells were transfected with a commercial siRNA smart pool of three oligonucleotides (sip53) transiently targeting p53 (Santa Cruz Biotech, Dallas, TX, USA; sc-29435) ~~a targeting siRNA (see figure legends)~~ and a non-targeting siRNA as negative control, using Lipofectamine 2000 (Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. The ectopic expression of mutant p53 was carried out transfecting pcDNA3-mutp53R273H expression vector, or its relative mock vector (pcDNA3). After 6 hours of transfection, complexes were removed and cells were maintained in DMEM for 48 hours.

2.3 Lentivirus cell transduction

To silence R273H mutp53 expression in MeWo cells, we used plasmid pLKO.1 puro-vector encoding TP53-shRNA (TRCN0000003756; Sigma-Aldrich) indicated as p53-SH1. As negative control we used a non-target shRNA control (SHC016; Sigma-Aldrich) indicated as p53-NT. To generate viral particles, 293FT cells (Thermo Fisher) were transfected using pLKO.1 shRNA DNA vector together with ViraPower Lentiviral Packaging Mix (pLP1, pLP2 and pLP/VSV-G) (Thermo Fisher). Seventy-two hours later, viral supernatant was collected and transducing units per ml of supernatant were determined by limiting dilution titration in cells. A Multiplicity Of Infection (MOI) of 5 to 1 (5 transducing viral particles to 1 cell) was used for transducing cells using pPolybrene (Sigma-Aldrich) at a final concentration of 8 µg/ml to increase transduction efficiency. Twenty-four hours after transduction, puromycin selection (2 µg/ml) was performed for 48 h and mutant TP53-silenced cells were used for experiments.

2.34 Cellular treatment with hydrogen peroxide (H_2O_2)

MeWo and A375 cells ($4x10^5$) were seeded on a 6-well plate. After 24 hours, cells were treated with 50 µM H_20_2 (30% W/V) (Applichem) for 24 hours. Cells were harvested, RNA was extracted and the mRNA expression levels were determined by qPCR, as detailed below.

2.45 Apoptotic assay

Cells were seeded in 96-well plate ($4x10^3$ cells/well) and the day after were transfected with the indicated constructs (see figure legends) and further incubated for 48 hours. At the end of the treatments, cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature, washed twice with PBS and stained with annexin V/FITC (Bender MedSystem) in binding buffer (10mM HEPES/NaOH pH 7.4, 140mM NaCl, and 2.5mM $CaCl_2$) for 10 min at room temperature in the dark. Cells were then washed with binding buffer and fluorescence was measured using a multimode plate reader (Ex 485 nm and Em 535nm) (GENios Pro, Tecan). The values were normalized on cell proliferation by Crystal Violet assay.

2.~~5~~6~~3~~ *Cell proliferation assay*

Cells were seeded in 96-well plates and the day after were incubated with various compounds at the indicated conditions or transfected with the indicated constructs (see figure legends). At the end of the treatments, cell growth was measured by Crystal Violet assay (Sigma-Aldrich) according to the manufacturer's protocol, and absorbance was measured by spectrophotometric analysis (A_{595}nm).

2.~~6~~7~~4~~ *Analysis of intracellular* H_2O_2 ~~*ROS*~~

To analyze H_2O_2 production, the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Eugene, Oregon) 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 $CaCl_2$, 1.22 mM $MgSO_4$, 5.5 mM glucose, 5.7 mM sodium phosphate, pH 7.4) and the reaction mixture was added to the cells. Fluorescence measurement was recorded at times 0, 15 and 30 min on a FLx800 microplate fluorescence reader (Bio-Tek) set at excitation and emission wavelengths of 571 nm and 585 nm, respectively. Values were normalized per number of viable cells determined by Crystal Violet assay.

2.~~7~~8~~5~~ *Real-time quantitative PCR (qPCR)*

After 48 hours of transfection, total RNA was isolated from cultured cells using TRI-Reagent isolation reagent (Sigma) following the manufacturer's protocol. For each sample, 1 µg of RNA was reverse transcribed to cDNA at 42°C for 60 min with 25 U MuLV reverse transcriptase in a retrotranscription reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl2, 2.5 µM random hexamers, 10 U RNase inhibitor and 500 µM dNTP. qPCR was performed in triplicate samples using SYBR Green technology on a LightCycler 480 System II

thermal cycler (Roche Diagnostics, Basel, Switzerland). The amplification program consisted of a preincubation step for denaturation of 5 min at 95°C, followed by 45 cycles consisting of a denaturation step (10 s, 95°C), an annealing step (10 s, temperature depending on primers), and an elongation step (12 s, 72°C). The Ct values of the real-time PCR were analyzed using the GenEX Standard Software (Multi-DAnalises, Sweden). Genes, primers and temperatures for the annealing step are specified in **Table 1**.

2.~~8~~9~~6~~ Western Blotting

After 48 hours of transfection, 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 leupetin, 0.01 mM pepstatin, 2 mM PMSF, 1 mM NaF and 1 mM Na_3VO_4) and sonicated at 40% amplitude for 7 seconds three times (VibraCell 7185). Samples were then centrifuged at 14000x*g* for 10 min at 4°C. Protein content (supernatant) was determined with the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific).

Twenty-five micrograms of protein were resolved on a 15% SDS-PAGE gel and electrotransferred to 0.22 µm nitrocellulose membranes using the Trans-blot® Turbo™ transfer system (Bio-Rad). Membranes were blocked in 5% non-fat powdered milk in TBS with 0.05% Tween for 1 h. Antisera against p53 (#sc-263), MnSOD (#sc-30080; Santa Cruz Biotechnology, CA, USA), SIRT3 (#2526; Cell Signaling, MA, USA), acetylated (K68) MnSOD (ab137037; Abcam, OR, USA), and alpha-tubulin (#CP06; MerkMillipore, Darmstadt, Germany) were used as primary antibodies. Protein bands were visualized using Immun-Star® Western C® Kit reagent (Bio-Rad). The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and results were analyzed with Quantity One Software (Bio-Rad).

2.~~10~~9~~7~~ MnSOD enzymatic activity

Cells were harvested after 48 hours of transfection by scraping them in 200 µL of STE buffer (16.4 Tris HCl pH 7.4, 250 mM sucrose, 3.59 mM Trizma-Base, 2 mM EDTA, 40 mM KCl). Cells were disrupted by sonication at 40% amplitude for 7 s three times and centrifuged at 600x*g* for 10 min at 4°C. Protein content (supernatant) was determined by BCA assay kit. MnSOD activity was determined by following the reduction of cytochrome c by measuring the absorbance at 550 nm, and adding 1 mM KCN to inhibit CuZnSOD activity, as described before [14].

2.11~~0~~8 Statistical analysis

The Statistical Program for the Social Sciences software for Windows (SPSS, version 21.0; 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 p53 knockdown were assessed using the ANOVA analysis or the Student's t-test and statistical significance was set at ~~P~~p <0.05.

3. Results

3.1 Mutant p53 stimulates MnSOD by a ROS-dependent mechanism

To study the functional role of GOF mutant p53 in the regulation of the antioxidant MnSOD, we first analyzed the mRNA expression level and the activity of the enzyme in A375 and MeWo melanoma cell lines expressing wild type p53 and mutant $p53^{E258K}$, respectively. Cancer cells with mutant p53 had the endogenous level of both MnSOD mRNA expression (**Figure 1A**) and MnSOD activity (**Figure 1B**) significantly higher than cells with wild type *TP53* gene, suggesting a possible involvement of mutant p53 in the stimulation of the enzyme. When wild-type p53 A375 cells were knocked-down for p53 expression by using liposome-mediated transient transfection assay, the level of MnSOD mRNA and protein remained unchanged. Conversely, MnSOD expression was significantly decreased after knock-down of mutant p53 in MeWo cells (**Figure 2A**). We further strengthened these data through lentivirus-mediated transduction and qPCR analysis of MnSOD mRNA using a different sequence to knock-down mutant p53 expression (p53-SH1) or its negative control (p53-NT) in MeWo cells (**Supplementary Figure 1**). Moreover, ~~W~~we ~~further~~ investigated whether MnSOD stimulation may be considered a cytoprotective response of the cells to the enhanced production of ROS by mutant p53. It has been described that mutant p53, in addition to induce oncogenic functions, can bind to wild-type p53 as heterodimers acting as dominant negative regulators of wild-type p53 functionality [15]. Thus, we overexpressed in wild-type p53 A375 cells the $mutp53^{R273H}$ isoform, which has oncogenic activity such as the $mutp53^{E258K}$ isoform expressed in MeWo cells. We observed that the ectopic expression of mutant p53 increased ~~ROS~~ H_2O_2 production and MnSOD expression, and that the addition of the radical scavenger N-acetyl-L-cysteine (NAC) reversed both H_2O_2 ~~ROS~~ (**Figure 2B**) and MnSOD induction (**Figure 2C**). The control of p53 overexpression is shown in the figure 2C (lower panel). In addition, we demonstrated that MnSOD mRNA level was induced after treatment with exogenous H_2O_2 in mutant p53 MeWo cells, while it remained unchanged in wild-type p53 A375 cells (**Figure 2D**), suggesting that pro-oxidant conditions induced by mutant p53 can promote MnSOD induction.

Altogether ~~T~~these data indicate that MnSOD stimulation by mutant p53 is due to ROS increase, acting as a cytoprotective response of the cell to the enhanced ROS production likely to maintain oxidative stress below the cytotoxicity threshold.

3.2 Mutant p53 increases SIRT3 and decreases acetylated MnSOD

Notably, MnSOD contains specific lysine residues, which could be targets of reversible acetylation/deacetylation. In particular, MnSOD is acetylated at lysine 68 (K68) resulting in the enzyme activity decrease and SIRT3, a primary deacetylase localized to the mitochondria, can stimulate MnSOD activity by deacetylation to scavenge ROS [16,17]. We demonstrate that knocking-down mutant p53 in MeWo cells decreased the expression levels of both mRNA and protein of SIRT3, while wild-type p53 silencing in A375 cells did not change SIRT3 expression (**Figure 3A**). Moreover, mutp53^{R273H} overexpression increased SIRT3 expression in A375 cells (Figure 3A). These data indicate that mutant p53 isoforms acquired the capability to induce SIRT3 expression in melanoma cells and are further confirmed by lentivirus-mediated transduction and qPCR analysis of SIRT3 mRNA using a different sequence to knock-down mutant p53 expression (p53-SH1) or its negative control (p53-NT) in MeWo cells (Supplementary Figure 1). ~~These data indicate that mutant p53 isoforms acquired the capability to induce SIRT3 expression in melanoma cells.~~ In addition, we demonstrated that SIRT3 mRNA level was induced after treatment with exogenous H_2O_2 in mutant p53 MeWo cells, while it remained unchanged in wild-type p53 A375 cells (**Figure 3B**), suggesting that pro-oxidant conditions induced by mutant p53 can promote SIRT3 induction as well as MnSOD. To demonstrate that SIRT3 is a regulator of MnSOD acetylation and activity also in our system, we knocked-down SIRT3 expression by siRNA (**Supplementary Figure 2~~1~~**). Accordingly, SIRT3 silencing increased the level of MnSOD K68 acetylation (**Figure 3C~~B~~**), and MnSOD activity was reduced by SIRT3 knock-down and increased by SIRT3 overexpression (**Figure 3D~~C~~**). To highlight the regulation of MnSOD by mutant p53 we analyzed the acetylated/total MnSOD ratio after p53 regulation. **Figure 4** shows that wild-type p53

knock-down did not alter this ratio, while mutp53^{R273H} overexpression decreased the acetylated/total MnSOD ratio in A375 cells. Accordingly, mutant p53 knock-down increased the acetylated/total MnSOD ratio in MeWo cells (Figure 4). Altogether these data suggest that mutant p53 induces SIRT3 deacetylase and enhances MnSOD activity by deacetylation.

3.3 Mutant p53 enhances MnSOD activity by SIRT3 moderating ROS production

In accordance with the previous results, mutp53^{R273H} overexpression in A375 cells increased MnSOD activity (**Figure 5A**). Notably, the decrease of MnSOD activity by mutant p53 knock-down was reverted by overexpression of SIRT3 in MeWo cells, while wild-type p53 knock-down in A375 cells was ineffective (**Figure 5B**). Altogether these data suggest that mutant p53 increases MnSOD activity by inducing SIRT3-mediated MnSOD deacetylation. To investigate the ~~functional~~ role of MnSOD stimulation by mutant p53 on cancer cell growth ~~of MnSOD stimulation by mutant p53~~, we assessed H_2O_2 ~~ROS~~ production, apoptosis and cell proliferation. Functionally, ~~**Figure 5C shows that**~~ mutp53^{R273H} overexpression induced H_2O_2 ~~ROS~~ production **(Figure 5C)**, reduced apoptosis **(Figure 5D)** and stimulated cancer cell proliferation **(Figure 5E)**, ~~–~~supporting ~~an~~ the oncogenic role of mutp53-depedent ROS production as we previously reported in other cancer cell models [18]. Remarkably, in MnSOD knocking-down conditions, mutant p53^{R273H} further enhanced intracellular H_2O_2 ~~ROS~~ level and recovered both anti-apoptotic and hyperproliferative events ~~mutp53-dependent cell hyperproliferation~~ (F~~f~~igures 5C-5E). Altogether these~~Our~~ data suggest that MnSOD stimulation by mutant p53 is a mechanism that cancer cells adopt to moderate the pro-oxidant function of mutant p53 in order to avoid the excessive ROS production and its related cytotoxic effects, thus sustaining the oncogenic functions of mutant p53 isoforms in cancer.

A schematic representation of the molecular mechanisms identified in this study is provided in **Figure 6**. Overall, it emerges that mutant p53 stimulates MnSOD expression and activity by SIRT3-mediated deacetylation. Functionally, MnSOD stimulation contrasts with the induction of

ROS production through various mechanisms driven by mutant p53 previously described and can be considered a key mechanism to protect cancer cells from excessive ROS production.

4. Discussion

The majority of tumor-associated p53 mutations, particularly those defined as mutational "hotspots" occur within the DNA binding domain (DBD) of p53 [7]. Mutant p53 can engage in protein-protein interactions with a growing number of transcription factors or repressors, often being recruited to binding sites of those factors on chromatin, and modulate their transcriptional output both positively and negatively. Thus, mutant p53 isoforms can exert profound effects on gene expression patterns, and many of those genes are associated in various ways with cell proliferation and chemoresistance [19,20], alterations of energy metabolism [21–23], counteraction of autophagy [24,25] and alterations of cancer microenvironment [26,27], in line with the oncogenic effects of mutant p53 isoforms [28]. Concerning ROS, contrarily to the antioxidant functions of the wild-type counterpart, several evidence demonstrated that mutant p53 isoforms induce pro-oxidant conditions. Kalo *et al.* elucidated that mutp53^{R273H} interferes with the antioxidant function of NRF2 [29]. Boudreau *et al.* showed that mutant p53 proteins enhance the expression of the NADPH oxidase 4 NOX4, resulting in an increase of intracellular ROS levels, which sustains an invasive phenotype of breast cancer cells [30]. Khromova *et al.* demonstrated that p53 hotspot mutants increase intracellular ROS level stimulating angiogenesis and accelerating cancer growth in colon carcinoma xenografts [31]. Some studies also revealed that mutant p53 proteins suppress the expression of SLC7A11, a key component of the cystine/glutamate antiporter system xC^-, diminishing glutathione synthesis and resulting in redox imbalance [32,33]. Recently, we identified a novel mechanism by which mutant p53 proteins can stimulate their oncogenic pro-oxidant conditions through the inhibition of antioxidant sestrins (SESNs) and of the SESN1:AMPK complex, resulting in the down-regulation of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha/uncoupling protein 2 (PGC-1α/UCP2) axis, stimulating mitochondrial $O_2^{-}\cdot$ production without damaging mitochondrial DNA [18]. Some studies unveil that ROS play exceptional relevance in the development and progression of tumors being involved in the main features of aggressive cancer cell behavior, including genome instability, cellular hyper-

proliferation, epithelial-mesenchymal transition, invasion and metastasis [34]. However, the role of ROS in cancer cell biology is highly contextual and dependent on the nature of the stress, tumor tissue and stage [35]. Indeed, despite they can stimulate tumorigenesis and cancer development, a severe increase in ROS level may induce cell death following a non-specific injury of macromolecules and cellular organelles [36]. For instance, ROS can induce DNA damage and, consequently, a network of events collectively termed as the DNA damage response (DDR) is activated. This response includes DNA damage recognition, activation of checkpoints, cell cycle arrest, and eventually final outcomes of repair, apoptosis and immune clearance [37]. Functionally, the alternative reading frame (ARF) tumour suppressor protein has been recognized as a sensor of oxidative stress, acting as a barrier to cancer development [38]. In this context, Velimezi *et al.* intriguingly discovered a functional interplay between the DNA-damage-response kinase ATM and ARF tumour suppressor protein in human cancer [39].

In the present study, we describe a novel antioxidant mutp53-dependent mechanism by which cancer cells can maintain the ROS enhancement below a cytotoxic threshold. In fact, although the overall effect of mutant p53 is pro-oxidant, the induction of MnSOD allows to moderate this outcome. It might be hypothesized that the moderation of ROS production by MnSOD induction could be a mechanism for oncogenic mutant p53 isoforms to avoid the stimulation of cell death signals triggered by DDR, such as ARF induction, thus supporting cancer progression. We here reveal that mutant p53 induces mRNA and protein expression, as well as the enzymatic activity of MnSOD. The increase of MnSOD gene expression by mutant p53 may be due to various mechanisms, including the induction of key MnSOD gene regulators as c-Myc or NF-κB, which have been previously demonstrated to be stimulated by mutant p53 [40,41]. Notably, MnSOD is a critical regulator of tumour cell metabolism since its upregulation sustains aerobic glycolysis (named Warburg effect) [42]. Therefore, the positive regulation of MnSOD that we described may serve as oncogenic mechanism by which cancer bearing mutant p53 proteins promote the metabolic shift towards glycolysis to lead tumor progression [43]. In addition, MnSOD is considered a crucial detoxifying mitochondrial enzyme which can be induced by ROS increase [44], thus contributing to balance oxidant

conditions. This is in line with our results demonstrating that NAC addition revertsed ~~the~~ mutp53-mediated MnSOD induction and that the exogenous addition of H_2O_2 increases MnSOD expression in mutant p53 cancer cells, supporting the concept of MnSOD as a key mechanism to protect cancer cells from excessive ROS production. Furthermore, we also demonstrate that mutant p53 or the exogenous addition of H_2O_2 induce~~s~~ the expression of SIRT3, the major deacetylase in mitochondria, which plays a crucial role in modulating ROS and limiting the oxidative damage in cellular components. SIRT3 targets different enzymes which regulate mitochondrial metabolism and participate in ROS detoxification, such as the complexes of the respiratory chain, the isocitrate dehydrogenase, as well as MnSOD [45]. We here report that mutant p53 can induce MnSOD deacetylation stimulating its enzymatic activity. These data further support the induction at different levels of MnSOD to moderate ROS enhancement. In conclusion, our results suggest that mutant p53 tightly regulates oxidative stress in cancer cells, stimulating SIRT3 and MnSOD to maintain ROS levels controlled to promote cell proliferation and survival. Therefore, patients with tumors bearing mutant *TP53* gene could benefit from a pro-oxidant therapeutic strategy targeting MnSOD. This might provide new therapeutic opportunities to be further considered for clinical studies in cancer patients bearing mutant *TP53* gene.

Acknowledgments

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Conflict of interest

All authors declare that they have no conflicts of interest.

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Figure legends

Figure 1. Mutant p53 induces MnSOD expression and activity. **A)** MnSOD mRNA levels were determined by qPCR in A375 (WT-p53) and MeWo (mutp53^{E258K}) melanoma cell lines. Student's t test: *p < 0.05. **B)** MnSOD enzymatic activity was measured spectrophotometrically as described in the Methods section. Student's t test: *p < 0.05.

Figure 2. MnSOD regulation by mutant p53 is ROS-dependent. **A)** A375 (WT-p53) and MeWo (mutp53^{E258K}) melanoma cells were transfected with control- or p53-siRNA, and MnSOD mRNA and protein levels were analyzed by qPCR and Western Blot. Protein levels were normalized to tubulin expression. Student's t test: *p < 0.05; ns: non-significant. **B)** A375 cells were transfected with R273H mutant-p53 expression vector or its relative mock vector, and treated concomitantly with 7 mM NAC for 24 h to determine H_2O_2 ~~ROS~~ production as described in the Methods section. Student's t test: *p < 0.05. **C)**~~–~~ A375 cells were transfected with R273H mutant-p53 expression vector or its relative mock vector, and treated concomitantly with 7 mM NAC for 24 h. Expression of MnSOD was analyzed by qPCR and p53 overexpression was confirmed by Western Blot. Student's t test: *p < 0.05. **D)** A375 and MeWo cells were treated with 50 µM H_2O_2 for 24 h. Expression of MnSOD mRNA was analyzed by qPCR. Student's t test: *p < 0.05.

Figure 3. Mutant p53 promotes MnSOD deacetylation by increasing SIRT3 levels. **A)** A375 (WT-p53) and MeWo (mutp53^{E258K}) cells were transfected with the indicated siRNA or expression vector and SIRT3 mRNA and protein levels were analyzed by qPCR and Western Blot. Protein levels were normalized to tubulin expression. Student's t test: *p < 0.05; ns: non-significant. **B)** A375 and MeWo cells were treated with 50 µM H_2O_2 for 24 h. Expression of SIRT3 mRNA was analyzed by qPCR. Student's t test: *p < 0.05. **C)** A375 cells were transfected with control- or SIRT3-siRNA and levels of MnSOD K68 acetylation were determined by Western Blot. Protein levels were normalized to tubulin expression. Student's t test: *p < 0.05. **D~~C~~)** A375 cells were transfected with

SIRT3 expression vector or its relative mock vector and MnSOD enzymatic activity was measured by the described spectrophotometric method. Student's t test: *p < 0.05.

Figure 4. Mutant p53 decreases the acetylated/total MnSOD ratio. A375 (WT-p53) and MeWo (mutp53^{E258K}) melanoma cells were transfected with the indicated siRNA or expression vector and levels of MnSOD and acetylated MnSOD (K68) were determined by Western Blot. Protein levels were normalized to tubulin expression and the ratio acetylated/total MnSOD was calculated. Student's t test: *p < 0.05; ns: non-significant.

Figure 5. Modulation of MnSOD by mutant p53 contributes to regulate ROS production and cell viability. **A)** A375 cells were transfected with R273H mutant-p53 expression vector or its relative mock vector and MnSOD enzymatic activity was evaluated spectrophotometrically. Student's t test: *p < 0.05. **B)** A375 and MeWo cells were transfected with the indicated siRNA or expression vector and MnSOD enzymatic activity was evaluated spectrophotometrically. Student's t test: *p < 0.05; ns: non-significant. **C-E)** A375 cells were transfected with R273H mutant-p53 expression vector or its relative mock vector and/or with MnSOD-targeting siRNA. H_2O_2 ~~ROS~~ production (C), apoptosis (D) and cell proliferation (E) were determined by using Amplex Red probe, annexinV-FITC probe, and crystal violet staining~~methods~~, respectively. ANOVA test: experimental groups that do not share the same letter are statistically different (~~-~~p < 0.05).

Figure 6. Model of the molecular mechanisms by which mutant p53 regulates MnSOD, ROS production and cell growth.

Supplementary Figure legends

Supplementary Figure 1. Mutant p53 induces the expression of MnSOD and SIRT3 mRNAs. MeWo cells were transduced with lentiviruses containing p53-SH1 vector for mutant p53 silencing or its non-targeting negative control (NT). Left panel: Western Blot was performed using 50 µg of

whole-protein extracts and probed with the indicated antibodies. The p53 expression was shown as control of p53 knock-down efficacy and the GAPDH expression was used as control of equal proteins loading. Right panel: MnSOD and SIRT3 mRNA levels were determined by qPCR. Student's t test: *p < 0.05.

Supplementary Figure 21. Confirmation of SIRT3 knock-down by SIRT3-siRNA. A375 cells were transfected with control- or SIRT3-siRNA and levels of SIRT3 were analyzed by Western Blot. Protein levels were normalized to tubulin expression. Student's t test: *p < 0.05.

Mutant p53 induces SIRT3/MnSOD axis to moderate ROS production in melanoma cells

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Running title: Mutant p53 induces MnSOD

Keywords: ROS; mutant p53; gain-of-function; cancer; SIRT3; MnSOD; melanoma

Abstract

The *TP53* tumor suppressor gene is the most frequently altered gene in tumors and mutant p53 isoforms can acquire oncogenic properties referred to as gain-of-function (GOF). In this study, we used wild-type (A375) and mutant p53 (MeWo) melanoma cell lines to assess the regulation of the mitochondrial antioxidant manganese superoxide dismutase (MnSOD) by mutant p53. The effects of mutant p53 were evaluated by qPCR, immunoblotting, enzyme activity assay, cell proliferation assay, reactive oxygen species (ROS) assay after cellular transfection. We demonstrate that mutant p53 induces MnSOD expression, which is recovered by the ROS scavenger N-acetyl-L-cysteine. This suggests MnSOD induction as a defense mechanism of melanoma cells to counterbalance the pro-oxidant conditions induced by mutant p53. We also demonstrate that mutant p53 induces the expression of Sirtuin3 (SIRT3), a major mitochondrial NAD^+-dependent deacetylase, stimulating MnSOD deacetylation and enzymatic activity. Indeed, the restoration of SIRT3 reverses MnSOD activity decrease by mutant p53 knock-down. Finally, MnSOD knock-down further enhances mutant p53-mediated ROS increase, contracting mutp53-dependent cell hyperproliferation. This indicates that SIRT3 and MnSOD act to maintain ROS levels controlled to promote cell proliferation and survival, providing new therapeutic opportunities to be further considered for clinical studies in cancer patients bearing mutant *TP53* gene.

1. Introduction

Cutaneous melanoma is one of the most aggressive and lethal types of skin cancer that has its origins in melanocytes, especially among the white population. Its incidence is expected to grow over the next few decades due to the increasing trends in sun exposure [1]. UV radiation triggers reactive oxygen species (ROS) production, which leads to oxidative damage that may induce carcinogenesis [2]. Melanoma progression depends on many factors, especially the accumulation of genetic mutations that promote dissemination to other organs allowing cell survival to metastatic sites, in particular leading brain secondary tumors [3]. The tumor suppressor p53 can be considered the main checkpoint system of the cells, protecting them from oxidative stress via the induction of a number of antioxidant genes [4,5]. It is also a key regulator of genome integrity and cellular homeostasis through an intricate network of p53-dependent pathways, resulting in cell-cycle arrest, damage repair, senescence, apoptosis or modulation of energy metabolism [6]. However, mutations in the *TP53* gene can occur in over 50% of the human cancers and in 35% of sporadic cases of skin cancer [7]. Most of them are missense mutations that result in the expression of mutant isoforms of the p53 protein, which can acquire new biological properties referred to as gain-of-function (GOF) [8]. In addition to the loss of the tumor suppressor function of wild-type p53, GOF mutant p53 proteins contribute to the maintenance and stimulation of cancer growth through the acquisition of various oncogenic functions [9], compromising the response to anticancer treatments [10]. Different models have been proposed to explain the GOF activities of mutant p53, including binding and inactivation of the p53 family members p63 and p73 [11], modulation of the activity of a number of transcription factors, or inactivation of DNA damage molecular sensors [12,13]. It is emerging that mutant p53 proteins, contrarily to their wild-type p53 counterpart, fail to exert antioxidant properties rather sustaining a controlled increase of intracellular ROS, which favors cancer progression. In this study, we have investigated a novel survival mechanism of cancer cells induced by mutant p53, which partially counterbalances the mutant p53-dependent ROS production. This oncogenic mechanism may allow cancer cells to moderate the level of ROS increased by mutant

p53 itself, enabling them to survive even in a highly stressful oxidative environment. Our data reveal for the first time that mutant p53 can increase the expression of the key antioxidant detoxifying enzyme manganese superoxide dismutase (MnSOD) and its activity by SIRT3-mediated deacetylation in melanoma cells, contributing to temper the level of ROS and preventing their cytotoxic effects.

2. Material and Methods

2.1 Reagents

Dulbecco's modified Eagle's medium (DMEM) high glucose was obtained from Gibco (Paisley, UK). The siRNA targeting p53 (sc-29435), the siRNA targeting SIRT3 (sc-61555), the siRNA targeting MnSOD (sc-41655), and the non-targeting siRNA (sc-37007) were purchased at Santa Cruz Biotechnology (CA, USA). SIRT3 expression vector (SC127342) and pCMV6-AC control vector (PS100020) were purchased from Origene (Rockville, MD, USA). Routine chemicals were obtained from Sigma-Aldrich (Barcelona, Spain), Panreac (Barcelona, Spain) and Bio-Rad Laboratories (Hercules, CA, USA).

2.2 Cell culture and liposome-mediated transient cell transfection

A375 (WT-p53) and MeWo (mutp53^{E258K}) melanoma cell lines were used for all experiments. Cells were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotics penicillin and streptomycin and maintained in a humidified atmosphere of 5% CO_2 and 37°C. Cells were routinely tested to confirm lack of mycoplasma infection. For siRNA transfection, $4x10^5$ cells were seeded in 6-well plates, and $8x10^3$ were seeded in 96-well plates. The next day, cells were transfected with a commercial siRNA smart pool of three oligonucleotides (sip53) transiently targeting p53 (Santa Cruz Biotech, Dallas, TX, USA; sc-29435) and a non-targeting siRNA as negative control, using Lipofectamine 2000 (Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. The ectopic expression of mutant p53 was carried out transfecting pcDNA3-mutp53R273H expression vector, or its relative mock vector (pcDNA3). After 6 hours of transfection, complexes were removed and cells were maintained in DMEM for 48 hours.

2.3 Lentivirus cell transduction

To silence R273H mutp53 expression in MeWo cells, we used plasmid pLKO.1 puro-vector encoding TP53-shRNA (TRCN0000003756; Sigma-Aldrich) indicated as p53-SH1. As negative control we used a non-target shRNA control (SHC016; Sigma-Aldrich) indicated as p53-NT. To generate viral particles, 293FT cells (Thermo Fisher) were transfected using pLKO.1 shRNA DNA vector together with ViraPower Lentiviral Packaging Mix (pLP1, pLP2 and pLP/VSV-G) (Thermo Fisher). Seventy-two hours later, viral supernatant was collected and transducing units per ml of supernatant were determined by limiting dilution titration in cells. A Multiplicity Of Infection (MOI) of 5 to 1 (5 transducing viral particles to 1 cell) was used for transducing cells using polybrene (Sigma-Aldrich) at a final concentration of 8 μg/ml to increase transduction efficiency. Twenty-four hours after transduction, puromycin selection (2 μg/ml) was performed for 48 h and mutant TP53-silenced cells were used for experiments.

2.4 Cellular treatment with hydrogen peroxide (H_2O_2)

MeWo and A375 cells ($4x10^5$) were seeded on a 6-well plate. After 24 hours, cells were treated with 50 μM H_20_2 (30% W/V) (Applichem) for 24 hours. Cells were harvested, RNA was extracted and the mRNA expression levels were determined by qPCR, as detailed below.

2.5 Apoptotic assay

Cells were seeded in 96-well plate ($4x10^3$ cells/well) and the day after were transfected with the indicated constructs (see figure legends) and further incubated for 48 hours. At the end of the treatments, cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature, washed twice with PBS and stained with annexin V/FITC (Bender MedSystem) in binding buffer (10mM HEPES/NaOH pH 7.4, 140mM NaCl, and 2.5mM $CaCl_2$) for 10 min at room temperature in the dark. Cells were then washed with binding buffer and fluorescence was measured using a multimode plate reader (Ex 485 nm and Em 535nm) (GENios Pro, Tecan). The values were normalized on cell proliferation by Crystal Violet assay.

2.6 Cell proliferation assay

Cells were seeded in 96-well plates and the day after were incubated with various compounds at the indicated conditions or transfected with the indicated constructs (see figure legends). At the end of the treatments, cell growth was measured by Crystal Violet assay (Sigma-Aldrich) according to the manufacturer's protocol, and absorbance was measured by spectrophotometric analysis (A_{595}nm).

2.7 Analysis of intracellular H_2O_2

To analyze H_2O_2 production, the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Eugene, Oregon) 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 $CaCl_2$, 1.22 mM $MgSO_4$, 5.5 mM glucose, 5.7 mM sodium phosphate, pH 7.4) and the reaction mixture was added to the cells. Fluorescence measurement was recorded at times 0, 15 and 30 min on a FLx800 microplate fluorescence reader (Bio-Tek) set at excitation and emission wavelengths of 571 nm and 585 nm, respectively. Values were normalized per number of viable cells determined by Crystal Violet assay.

2.8 Real-time quantitative PCR (qPCR)

After 48 hours of transfection, total RNA was isolated from cultured cells using TRI-Reagent isolation reagent (Sigma) following the manufacturer's protocol. For each sample, 1 μg of RNA was reverse transcribed to cDNA at 42°C for 60 min with 25 U MuLV reverse transcriptase in a retrotranscription reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM MgCl2, 2.5 μM random hexamers, 10 U RNase inhibitor and 500 μM dNTP. qPCR was performed in triplicate samples using SYBR Green technology on a LightCycler 480 System II

thermal cycler (Roche Diagnostics, Basel, Switzerland). The amplification program consisted of a preincubation step for denaturation of 5 min at 95°C, followed by 45 cycles consisting of a denaturation step (10 s, 95°C), an annealing step (10 s, temperature depending on primers), and an elongation step (12 s, 72°C). The Ct values of the real-time PCR were analyzed using the GenEX Standard Software (Multi-DAnalises, Sweden). Genes, primers and temperatures for the annealing step are specified in **Table 1**.

2.9 Western Blotting

After 48 hours of transfection, 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 leupetin, 0.01 mM pepstatin, 2 mM PMSF, 1 mM NaF and 1 mM Na_3VO_4) and sonicated at 40% amplitude for 7 seconds three times (VibraCell 7185). Samples were then centrifuged at 14000x*g* for 10 min at 4°C. Protein content (supernatant) was determined with the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific).

Twenty-five micrograms of protein were resolved on a 15% SDS-PAGE gel and electrotransferred to 0.22 µm nitrocellulose membranes using the Trans-blot® Turbo™ transfer system (Bio-Rad). Membranes were blocked in 5% non-fat powdered milk in TBS with 0.05% Tween for 1 h. Antisera against p53 (#sc-263), MnSOD (#sc-30080; Santa Cruz Biotechnology, CA, USA), SIRT3 (#2526; Cell Signaling, MA, USA), acetylated (K68) MnSOD (ab137037; Abcam, OR, USA), and alpha-tubulin (#CP06; MerkMillipore, Darmstadt, Germany) were used as primary antibodies. Protein bands were visualized using Immun-Star® Western C® Kit reagent (Bio-Rad). The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and results were analyzed with Quantity One Software (Bio-Rad).

2.10 MnSOD enzymatic activity

Cells were harvested after 48 hours of transfection by scraping them in 200 µL of STE buffer (16.4 Tris HCl pH 7.4, 250 mM sucrose, 3.59 mM Trizma-Base, 2 mM EDTA, 40 mM KCl). Cells were disrupted by sonication at 40% amplitude for 7 s three times and centrifuged at 600x*g* for 10 min at 4°C. Protein content (supernatant) was determined by BCA assay kit. MnSOD activity was determined by following the reduction of cytochrome c by measuring the absorbance at 550 nm, and adding 1 mM KCN to inhibit CuZnSOD activity, as described before [14].

2.11 Statistical analysis

The Statistical Program for the Social Sciences software for Windows (SPSS, version 21.0; 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 p53 knockdown were assessed using the ANOVA analysis or the Student's t-test and statistical significance was set at $p < 0.05$.

3. Results

3.1 Mutant p53 stimulates MnSOD by a ROS-dependent mechanism

To study the functional role of GOF mutant p53 in the regulation of the antioxidant MnSOD, we first analyzed the mRNA expression level and the activity of the enzyme in A375 and MeWo melanoma cell lines expressing wild type p53 and mutant $p53^{E258K}$, respectively. Cancer cells with mutant p53 had the endogenous level of both MnSOD mRNA expression (**Figure 1A**) and MnSOD activity (**Figure 1B**) significantly higher than cells with wild type *TP53* gene, suggesting a possible involvement of mutant p53 in the stimulation of the enzyme. When wild-type p53 A375 cells were knocked-down for p53 expression by using liposome-mediated transient transfection assay, the level of MnSOD mRNA and protein remained unchanged. Conversely, MnSOD expression was significantly decreased after knock-down of mutant p53 in MeWo cells (**Figure 2A**). We further strengthened these data through lentivirus-mediated transduction and qPCR analysis of MnSOD mRNA using a different sequence to knock-down mutant p53 expression (p53-SH1) or its negative control (p53-NT) in MeWo cells (**Supplementary Figure 1**). Moreover, we investigated whether MnSOD stimulation may be considered a cytoprotective response of the cells to the enhanced production of ROS by mutant p53. It has been described that mutant p53, in addition to induce oncogenic functions, can bind to wild-type p53 as heterodimers acting as dominant negative regulators of wild-type p53 functionality [15]. Thus, we overexpressed in wild-type p53 A375 cells the $mutp53^{R273H}$ isoform, which has oncogenic activity such as the $mutp53^{E258K}$ isoform expressed in MeWo cells. We observed that the ectopic expression of mutant p53 increased H_2O_2 production and MnSOD expression, and that the addition of the radical scavenger N-acetyl-L-cysteine (NAC) reversed both H_2O_2 (**Figure 2B**) and MnSOD induction (**Figure 2C**). The control of p53 overexpression is shown in the figure 2C (lower panel). In addition, we demonstrated that MnSOD mRNA level was induced after treatment with exogenous H_2O_2 in mutant p53 MeWo cells, while it remained unchanged in wild-type p53 A375 cells (**Figure 2D**), suggesting that pro-oxidant conditions induced by mutant p53 can promote MnSOD induction. Altogether these data indicate

that MnSOD stimulation by mutant p53 is due to ROS increase, acting as a cytoprotective response of the cell to the enhanced ROS production likely to maintain oxidative stress below the cytotoxicity threshold.

3.2 Mutant p53 increases SIRT3 and decreases acetylated MnSOD

Notably, MnSOD contains specific lysine residues, which could be targets of reversible acetylation/deacetylation. In particular, MnSOD is acetylated at lysine 68 (K68) resulting in the enzyme activity decrease and SIRT3, a primary deacetylase localized to the mitochondria, can stimulate MnSOD activity by deacetylation to scavenge ROS [16,17]. We demonstrate that knocking-down mutant p53 in MeWo cells decreased the expression levels of both mRNA and protein of SIRT3, while wild-type p53 silencing in A375 cells did not change SIRT3 expression (**Figure 3A**). Moreover, mutp53^{R273H} overexpression increased SIRT3 expression in A375 cells (Figure 3A). These data indicate that mutant p53 isoforms acquired the capability to induce SIRT3 expression in melanoma cells and are further confirmed by lentivirus-mediated transduction and qPCR analysis of SIRT3 mRNA using a different sequence to knock-down mutant p53 expression (p53-SH1) or its negative control (p53-NT) in MeWo cells (Supplementary Figure 1). In addition, we demonstrated that SIRT3 mRNA level was induced after treatment with exogenous H_2O_2 in mutant p53 MeWo cells, while it remained unchanged in wild-type p53 A375 cells (**Figure 3B**), suggesting that pro-oxidant conditions induced by mutant p53 can promote SIRT3 induction as well as MnSOD. To demonstrate that SIRT3 is a regulator of MnSOD acetylation and activity also in our system, we knocked-down SIRT3 expression by siRNA (**Supplementary Figure 2**). Accordingly, SIRT3 silencing increased the level of MnSOD K68 acetylation (**Figure 3C**), and MnSOD activity was reduced by SIRT3 knock-down and increased by SIRT3 overexpression (**Figure 3D**). To highlight the regulation of MnSOD by mutant p53 we analyzed the acetylated/total MnSOD ratio after p53 regulation. **Figure 4** shows that wild-type p53 knock-down did not alter this ratio, while mutp53^{R273H} overexpression decreased the acetylated/total MnSOD ratio in A375 cells.

Accordingly, mutant p53 knock-down increased the acetylated/total MnSOD ratio in MeWo cells (Figure 4). Altogether these data suggest that mutant p53 induces SIRT3 deacetylase and enhances MnSOD activity by deacetylation.

3.3 Mutant p53 enhances MnSOD activity by SIRT3 moderating ROS production

In accordance with the previous results, mutp53^{R273H} overexpression in A375 cells increased MnSOD activity (**Figure 5A**). Notably, the decrease of MnSOD activity by mutant p53 knock-down was reverted by overexpression of SIRT3 in MeWo cells, while wild-type p53 knock-down in A375 cells was ineffective (**Figure 5B**). Altogether these data suggest that mutant p53 increases MnSOD activity by inducing SIRT3-mediated MnSOD deacetylation. To investigate the role of MnSOD stimulation by mutant p53 on cancer cell growth, we assessed H_2O_2 production, apoptosis and cell proliferation. Functionally, mutp53^{R273H} overexpression induced H_2O_2 production (**Figure 5C**), reduced apoptosis (**Figure 5D**) and stimulated cancer cell proliferation (**Figure 5E**), supporting the oncogenic role of mutp53-depedent ROS production as we previously reported in other cancer cell models [18]. Remarkably, in MnSOD knocking-down conditions, mutant p53^{R273H} further enhanced intracellular H_2O_2 level and recovered both anti-apoptotic and hyperproliferative events (Figures 5C-5E). Altogether these data suggest that MnSOD stimulation by mutant p53 is a mechanism that cancer cells adopt to moderate the pro-oxidant function of mutant p53 in order to avoid the excessive ROS production and its related cytotoxic effects, thus sustaining the oncogenic functions of mutant p53 isoforms in cancer.

A schematic representation of the molecular mechanisms identified in this study is provided in **Figure 6**. Overall, it emerges that mutant p53 stimulates MnSOD expression and activity by SIRT3-mediated deacetylation. Functionally, MnSOD stimulation contrasts with the induction of ROS production through various mechanisms driven by mutant p53 previously described and can be considered a key mechanism to protect cancer cells from excessive ROS production.

4. Discussion

The majority of tumor-associated p53 mutations, particularly those defined as mutational "hotspots" occur within the DNA binding domain (DBD) of p53 [7]. Mutant p53 can engage in protein-protein interactions with a growing number of transcription factors or repressors, often being recruited to binding sites of those factors on chromatin, and modulate their transcriptional output both positively and negatively. Thus, mutant p53 isoforms can exert profound effects on gene expression patterns, and many of those genes are associated in various ways with cell proliferation and chemoresistance [19,20], alterations of energy metabolism [21–23], counteraction of autophagy [24,25] and alterations of cancer microenvironment [26,27], in line with the oncogenic effects of mutant p53 isoforms [28]. Concerning ROS, contrarily to the antioxidant functions of the wild-type counterpart, several evidence demonstrated that mutant p53 isoforms induce pro-oxidant conditions. Kalo *et al.* elucidated that mutp53^{R273H} interferes with the antioxidant function of NRF2 [29]. Boudreau *et al.* showed that mutant p53 proteins enhance the expression of the NADPH oxidase 4 NOX4, resulting in an increase of intracellular ROS levels, which sustains an invasive phenotype of breast cancer cells [30]. Khromova *et al.* demonstrated that p53 hotspot mutants increase intracellular ROS level stimulating angiogenesis and accelerating cancer growth in colon carcinoma xenografts [31]. Some studies also revealed that mutant p53 proteins suppress the expression of SLC7A11, a key component of the cystine/glutamate antiporter system xC^-, diminishing glutathione synthesis and resulting in redox imbalance [32,33]. Recently, we identified a novel mechanism by which mutant p53 proteins can stimulate their oncogenic pro-oxidant conditions through the inhibition of antioxidant sestrins (SESNs) and of the SESN1:AMPK complex, resulting in the down-regulation of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha/uncoupling protein 2 (PGC-1α/UCP2) axis, stimulating mitochondrial $O_2^{-}\cdot$ production without damaging mitochondrial DNA [18]. Some studies unveil that ROS play exceptional relevance in the development and progression of tumors being involved in the main features of aggressive cancer cell behavior, including genome instability, cellular hyper-

proliferation, epithelial-mesenchymal transition, invasion and metastasis [34]. However, the role of ROS in cancer cell biology is highly contextual and dependent on the nature of the stress, tumor tissue and stage [35]. Indeed, despite they can stimulate tumorigenesis and cancer development, a severe increase in ROS level may induce cell death following a non-specific injury of macromolecules and cellular organelles [36]. For instance, ROS can induce DNA damage and, consequently, a network of events collectively termed as the DNA damage response (DDR) is activated. This response includes DNA damage recognition, activation of checkpoints, cell cycle arrest, and eventually final outcomes of repair, apoptosis and immune clearance [37]. Functionally, the alternative reading frame (ARF) tumour suppressor protein has been recognized as a sensor of oxidative stress, acting as a barrier to cancer development [38]. In this context, Velimezi *et al.* intriguingly discovered a functional interplay between the DNA-damage-response kinase ATM and ARF tumour suppressor protein in human cancer [39]. In the present study, we describe a novel antioxidant mutp53-dependent mechanism by which cancer cells can maintain the ROS enhancement below a cytotoxic threshold. In fact, although the overall effect of mutant p53 is pro-oxidant, the induction of MnSOD allows to moderate this outcome. It might be hypothesized that the moderation of ROS production by MnSOD induction could be a mechanism for oncogenic mutant p53 isoforms to avoid the stimulation of cell death signals triggered by DDR, such as ARF induction, thus supporting cancer progression. We here reveal that mutant p53 induces mRNA and protein expression, as well as the enzymatic activity of MnSOD. The increase of MnSOD gene expression by mutant p53 may be due to various mechanisms, including the induction of key MnSOD gene regulators as c-Myc or NF-κB, which have been previously demonstrated to be stimulated by mutant p53 [40,41]. Notably, MnSOD is a critical regulator of tumour cell metabolism since its upregulation sustains aerobic glycolysis (named Warburg effect) [42]. Therefore, the positive regulation of MnSOD that we described may serve as oncogenic mechanism by which cancer bearing mutant p53 proteins promote the metabolic shift towards glycolysis to lead tumor progression [43]. In addition, MnSOD is considered a crucial detoxifying mitochondrial

enzyme which can be induced by ROS increase [44], thus contributing to balance oxidant conditions. This is in line with our results demonstrating that NAC addition reverts mutp53-mediated MnSOD induction and that the exogenous addition of H_2O_2 increases MnSOD expression in mutant p53 cancer cells, supporting the concept of MnSOD as a key mechanism to protect cancer cells from excessive ROS production. Furthermore, we also demonstrate that mutant p53 or the exogenous addition of H_2O_2 induce the expression of SIRT3, the major deacetylase in mitochondria, which plays a crucial role in modulating ROS and limiting the oxidative damage in cellular components. SIRT3 targets different enzymes which regulate mitochondrial metabolism and participate in ROS detoxification, such as the complexes of the respiratory chain, the isocitrate dehydrogenase, as well as MnSOD [45]. We here report that mutant p53 can induce MnSOD deacetylation stimulating its enzymatic activity. These data further support the induction at different levels of MnSOD to moderate ROS enhancement. In conclusion, our results suggest that mutant p53 tightly regulates oxidative stress in cancer cells, stimulating SIRT3 and MnSOD to maintain ROS levels controlled to promote cell proliferation and survival. Therefore, patients with tumors bearing mutant *TP53* gene could benefit from a pro-oxidant therapeutic strategy targeting MnSOD. This might provide new therapeutic opportunities to be further considered for clinical studies in cancer patients bearing mutant *TP53* gene.

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Conflict of interest

All authors declare that they have no conflicts of interest.

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Figure legends

Figure 1. Mutant p53 induces MnSOD expression and activity. **A)** MnSOD mRNA levels were determined by qPCR in A375 (WT-p53) and MeWo (mutp53^{E258K}) melanoma cell lines. Student's t test: *p < 0.05. **B)** MnSOD enzymatic activity was measured spectrophotometrically as described in the Methods section. Student's t test: *p < 0.05.

Figure 2. MnSOD regulation by mutant p53 is ROS-dependent. **A)** A375 (WT-p53) and MeWo (mutp53^{E258K}) melanoma cells were transfected with control- or p53-siRNA, and MnSOD mRNA and protein levels were analyzed by qPCR and Western Blot. Protein levels were normalized to tubulin expression. Student's t test: *p < 0.05; ns: non-significant. **B)** A375 cells were transfected with R273H mutant-p53 expression vector or its relative mock vector, and treated concomitantly with 7 mM NAC for 24 h to determine H_2O_2 production as described in the Methods section. Student's t test: *p < 0.05. **C)** A375 cells were transfected with R273H mutant-p53 expression vector or its relative mock vector, and treated concomitantly with 7 mM NAC for 24 h. Expression of MnSOD was analyzed by qPCR and p53 overexpression was confirmed by Western Blot. Student's t test: *p < 0.05. **D)** A375 and MeWo cells were treated with 50 μM H_2O_2 for 24 h. Expression of MnSOD mRNA was analyzed by qPCR. Student's t test: *p < 0.05.

Figure 3. Mutant p53 promotes MnSOD deacetylation by increasing SIRT3 levels. **A)** A375 (WT-p53) and MeWo (mutp53^{E258K}) cells were transfected with the indicated siRNA or expression vector and SIRT3 mRNA and protein levels were analyzed by qPCR and Western Blot. Protein levels were normalized to tubulin expression. Student's t test: *p < 0.05; ns: non-significant. **B)** A375 and MeWo cells were treated with 50 μM H_2O_2 for 24 h. Expression of SIRT3 mRNA was analyzed by qPCR. Student's t test: *p < 0.05. **C)** A375 cells were transfected with control- or SIRT3-siRNA and levels of MnSOD K68 acetylation were determined by Western Blot. Protein levels were normalized to tubulin expression. Student's t test: *p < 0.05. **D)** A375 cells were transfected with

SIRT3 expression vector or its relative mock vector and MnSOD enzymatic activity was measured by the described spectrophotometric method. Student's t test: *$p < 0.05$.

Figure 4. Mutant p53 decreases the acetylated/total MnSOD ratio. A375 (WT-p53) and MeWo (mutp53^{E258K}) melanoma cells were transfected with the indicated siRNA or expression vector and levels of MnSOD and acetylated MnSOD (K68) were determined by Western Blot. Protein levels were normalized to tubulin expression and the ratio acetylated/total MnSOD was calculated. Student's t test: *$p < 0.05$; ns: non-significant.

Figure 5. Modulation of MnSOD by mutant p53 contributes to regulate ROS production and cell viability. **A)** A375 cells were transfected with R273H mutant-p53 expression vector or its relative mock vector and MnSOD enzymatic activity was evaluated spectrophotometrically. Student's t test: *$p < 0.05$. **B)** A375 and MeWo cells were transfected with the indicated siRNA or expression vector and MnSOD enzymatic activity was evaluated spectrophotometrically. Student's t test: *$p < 0.05$; ns: non-significant. **C-E)** A375 cells were transfected with R273H mutant-p53 expression vector or its relative mock vector and/or with MnSOD-targeting siRNA. H_2O_2 production (C), apoptosis (D) and cell proliferation (E) were determined by using Amplex Red probe, annexinV-FITC probe, and crystal violet staining, respectively. ANOVA test: experimental groups that do not share the same letter are statistically different ($p < 0.05$).

Figure 6. Model of the molecular mechanisms by which mutant p53 regulates MnSOD, ROS production and cell growth.

Supplementary Figure legends

Supplementary Figure 1. Mutant p53 induces the expression of MnSOD and SIRT3 mRNAs. MeWo cells were transduced with lentiviruses containing p53-SH1 vector for mutant p53 silencing or its non-targeting negative control (NT). Left panel: Western Blot was performed using 50 μg of

whole-protein extracts and probed with the indicated antibodies. The p53 expression was shown as control of p53 knock-down efficacy and the GAPDH expression was used as control of equal proteins loading. Right panel: MnSOD and SIRT3 mRNA levels were determined by qPCR. Student's t test: *$p < 0.05$.

Supplementary Figure 2. Confirmation of SIRT3 knock-down by SIRT3-siRNA. A375 cells were transfected with control- or SIRT3-siRNA and levels of SIRT3 were analyzed by Western Blot. Protein levels were normalized to tubulin expression. Student's t test: *$p < 0.05$.

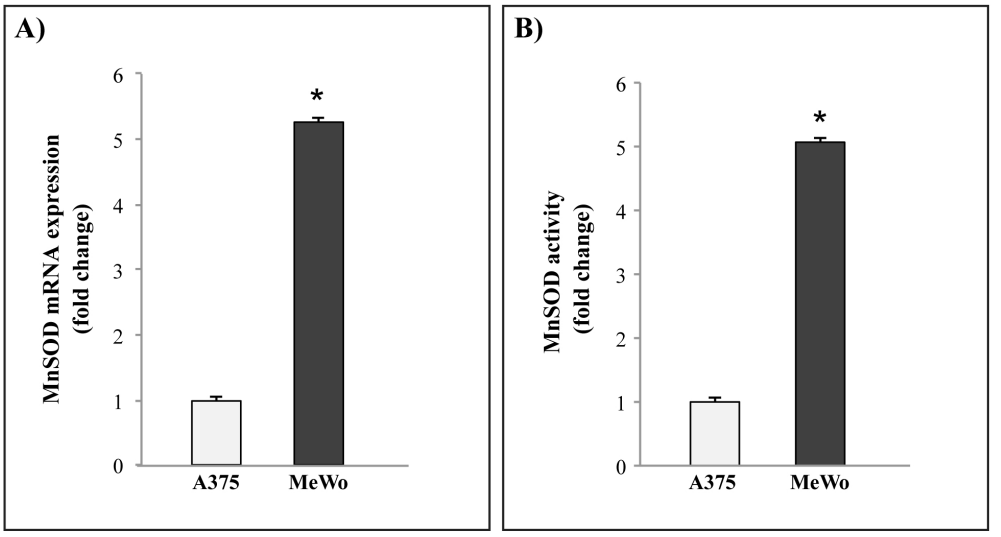

A)
MnSOD mRNA expression (fold change)
6
5
4
3
2
1
0
*
A375
MeWo
B)
MnSOD activity (fold change)
6
5
4
3
2
1
0
*
A375
MeWo

Fig. 1

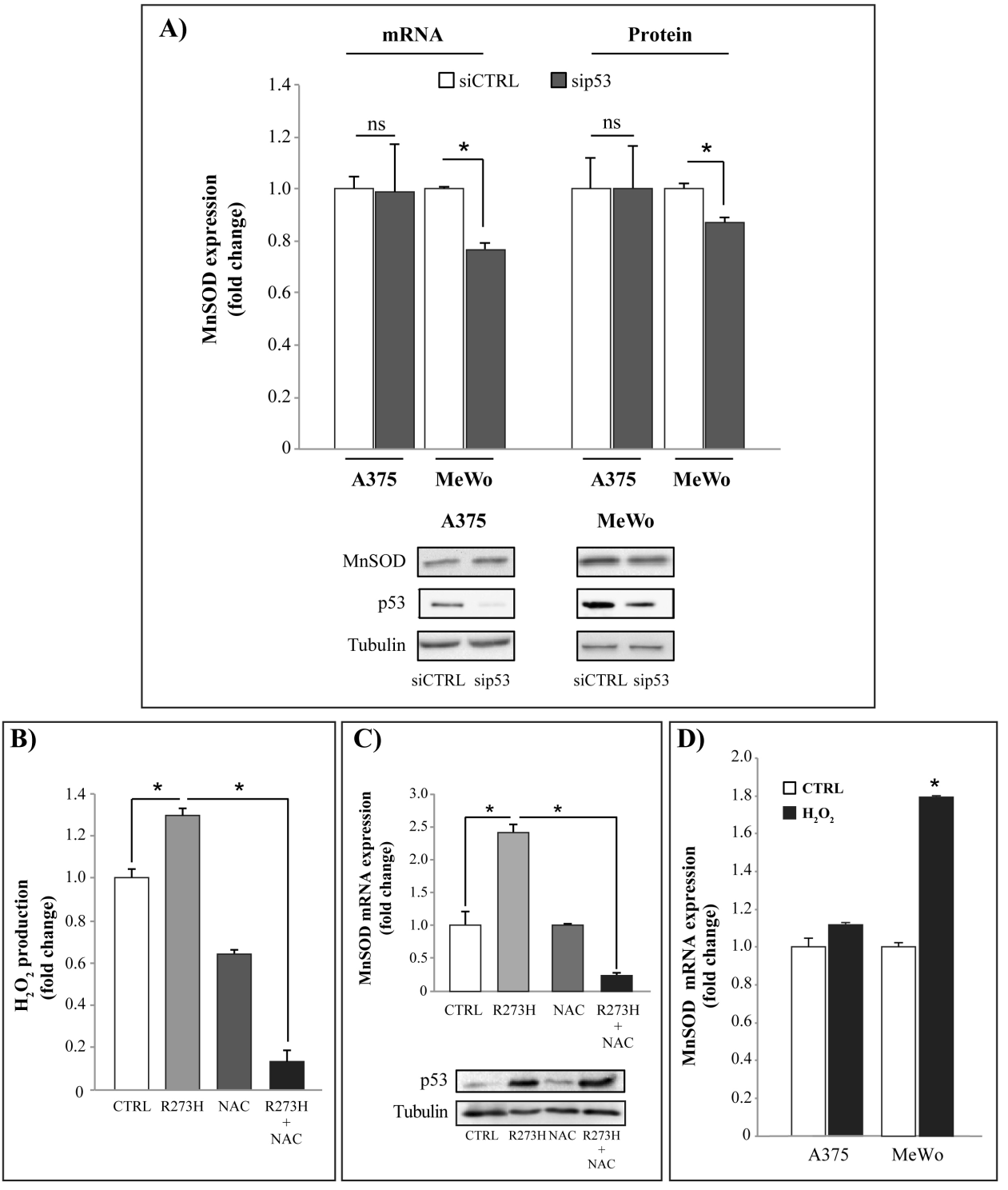

A)
mRNA
Protein
siCTRL
sip53
MnSOD expression (fold change)
ns
*
A375
MeWo
MnSOD
p53
Tubulin
siCTRL sip53
B)
H2O2 production (fold change)
CTRL
R273H
NAC
R273H + NAC
C)
MnSOD mRNA expression (fold change)
p53
Tubulin
D)
CTRL
H2O2
MnSOD mRNA expression (fold change)

Fig. 2

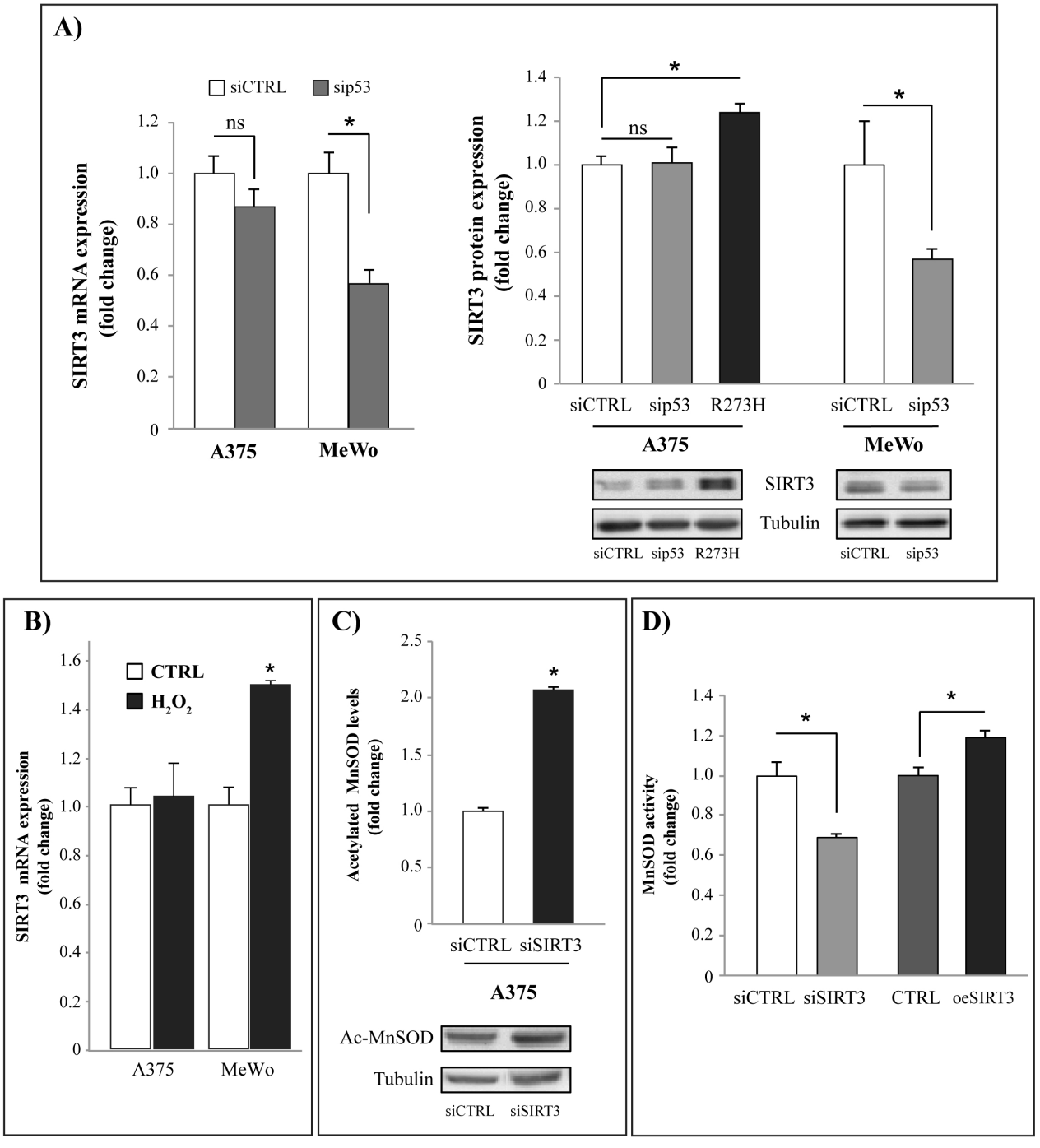

A)
siCTRL
sip53
SIRT3 mRNA expression (fold change)
ns
*
A375
MeWo
SIRT3 protein expression (fold change)
siCTRL
sip53
R273H
SIRT3
Tubulin
B)
CTRL
H2O2
SIRT3 mRNA expression (fold change)
C)
Acetylated MnSOD levels (fold change)
siSIRT3
Ac-MnSOD
D)
MnSOD activity (fold change)
CTRL
oeSIRT3

Fig. 3

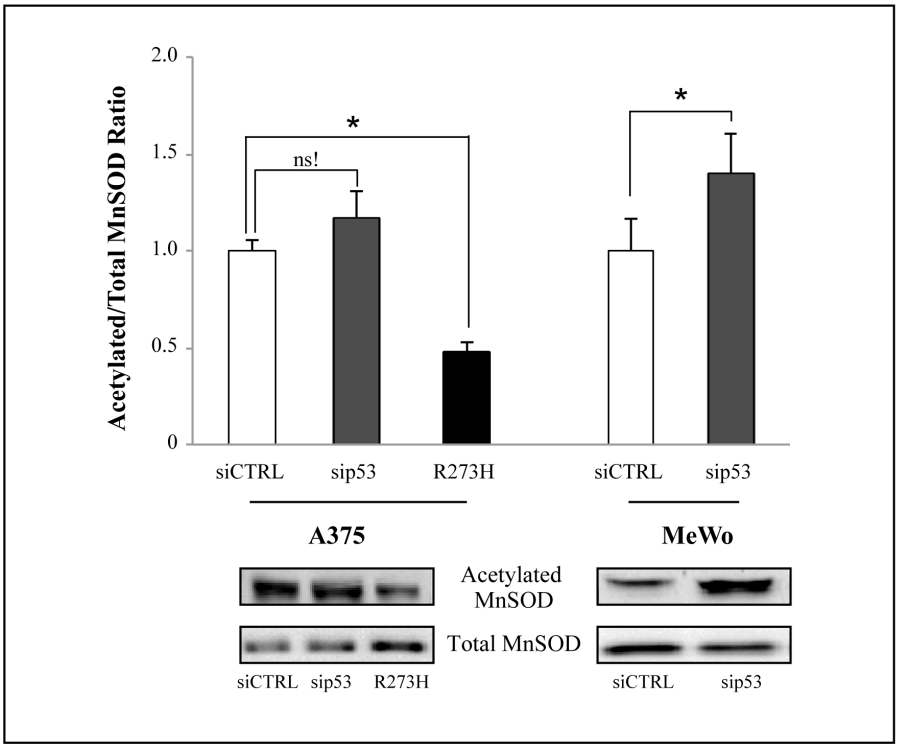

Acetylated/Total MnSOD Ratio
2.0
1.5
1.0
0.5
0
*
ns!
*
siCTRL
sip53
R273H
siCTRL
sip53
A375
MeWo
Acetylated MnSOD
Total MnSOD
siCTRL
sip53
R273H
siCTRL
sip53

Fig. 4

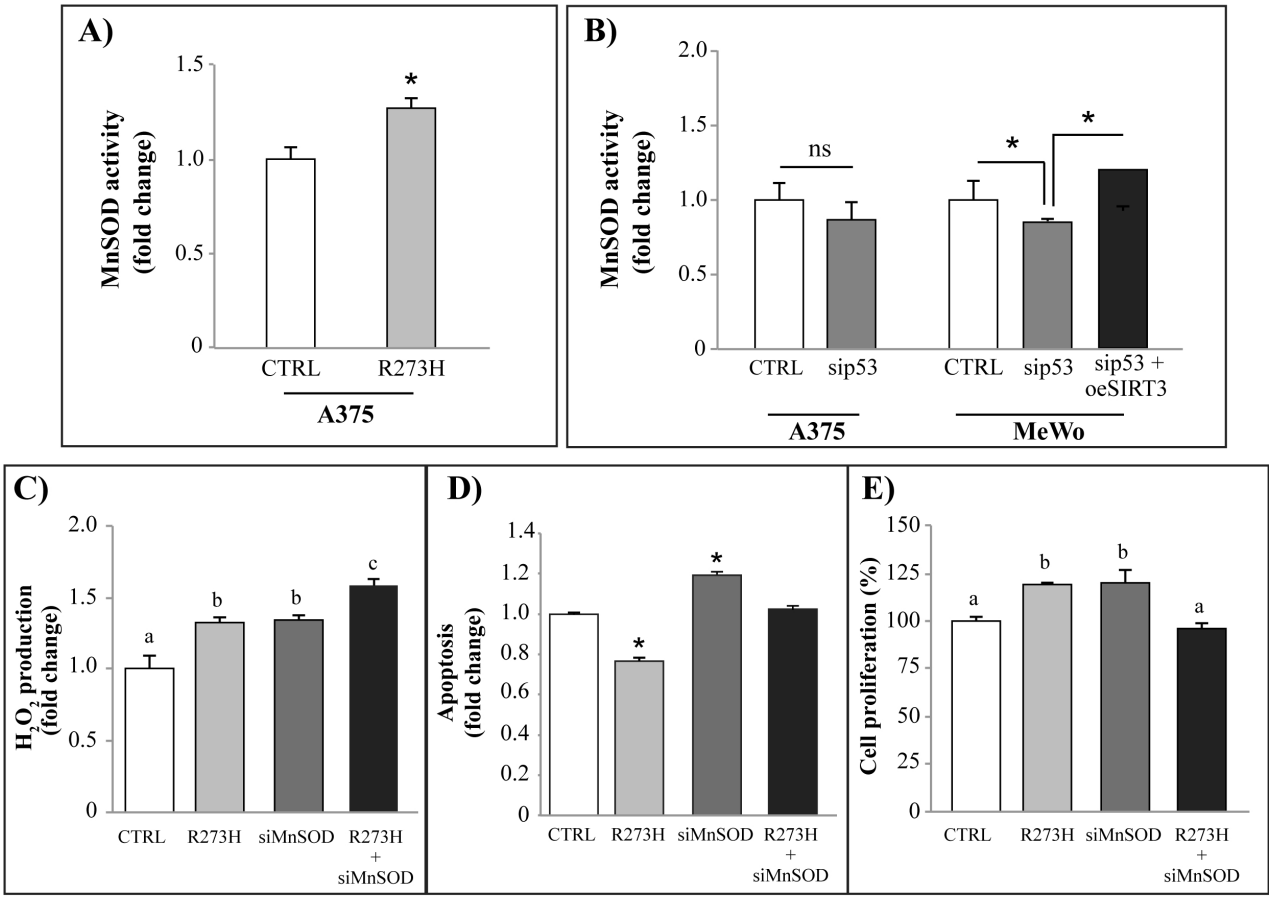

A)
MnSOD activity (fold change)
1.5
1.0
0.5
0
*
CTRL
R273H
A375
B)
MnSOD activity (fold change)
2.0
1.5
1.0
0.5
0
ns
*
*
CTRL
sip53
CTRL
sip53
sip53 + oeSIRT3
A375
MeWo
C)
H2O2 production (fold change)
2.0
1.5
1.0
0.5
0
a
b
b
c
CTRL
R273H
siMnSOD
R273H + siMnSOD
D)
Apoptosis (fold change)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
CTRL
R273H
siMnSOD
R273H + siMnSOD
E)
Cell proliferation (%)
150
125
100
75
50
25
0
a
b
b
a
CTRL
R273H
siMnSOD
R273H + siMnSOD

Fig. 5

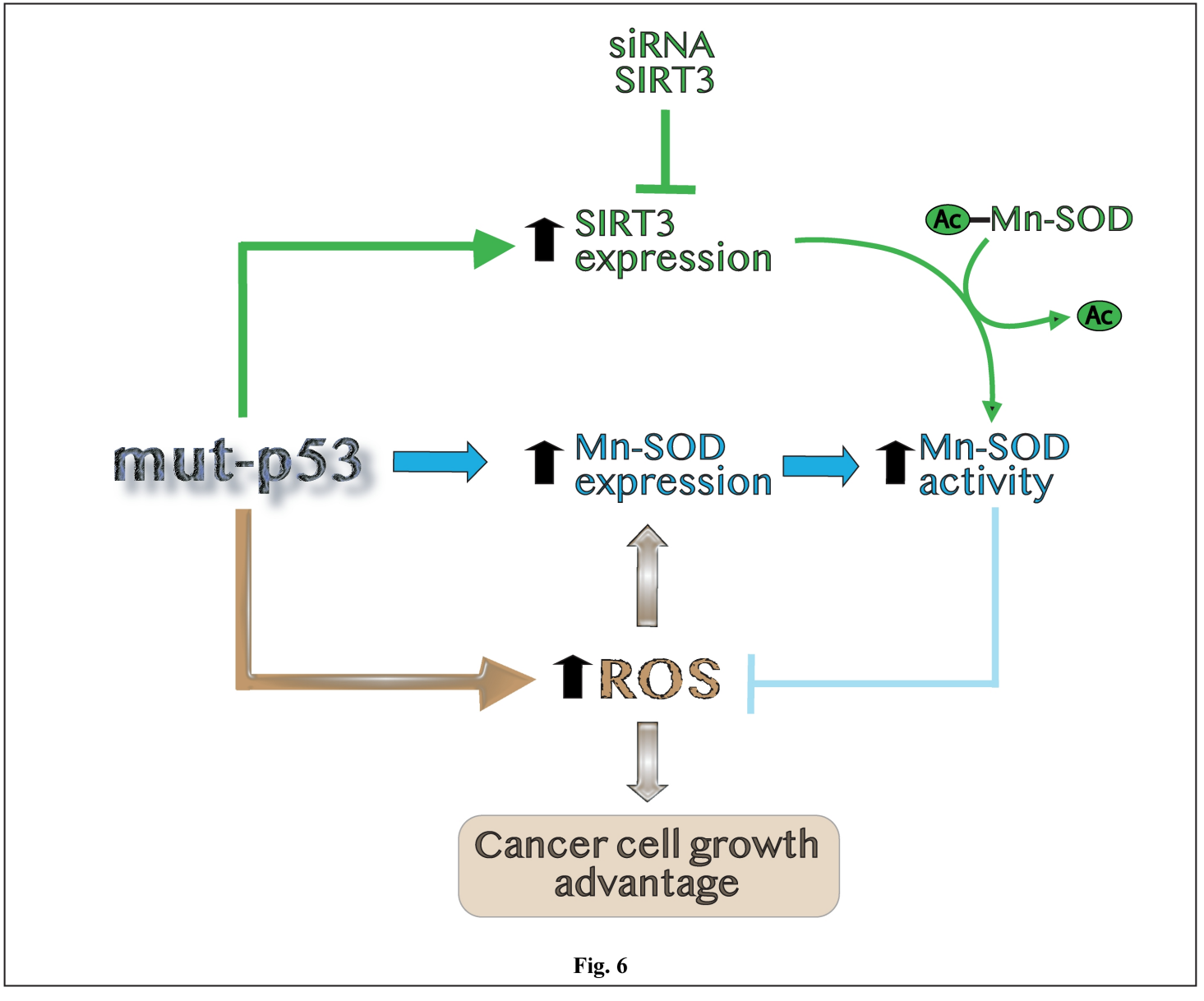
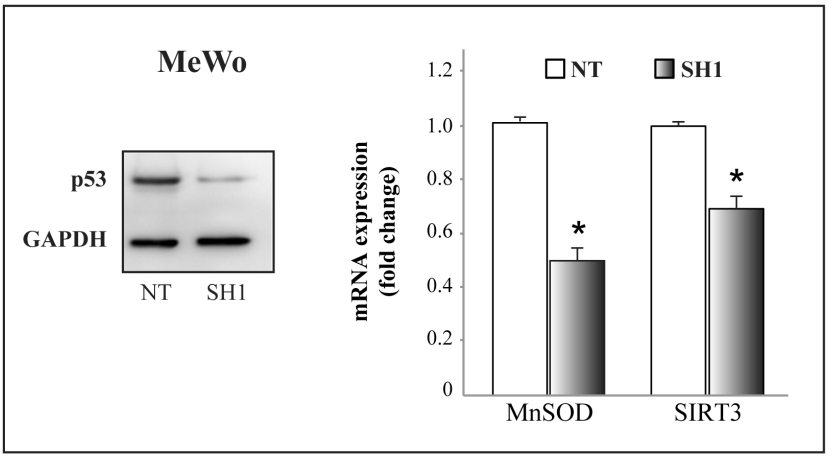
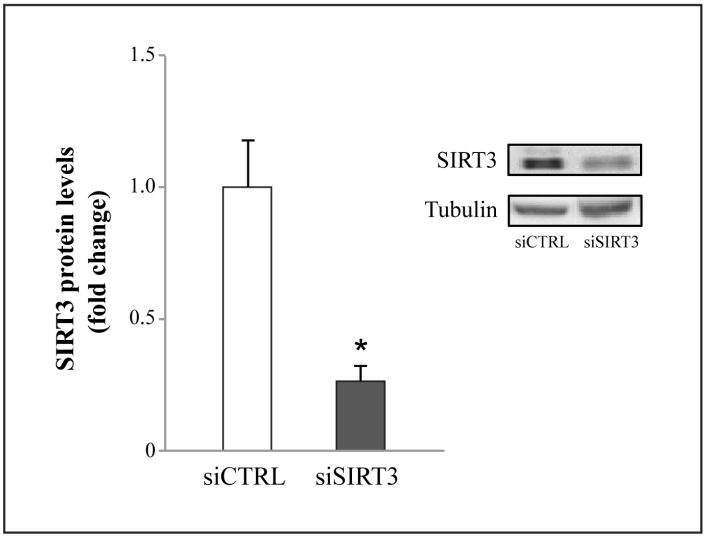

siRNA
SIRT3
SIRT3
expression
Ac
Mn-SOD
Ac
mut-p53
Mn-SOD
expression
Mn-SOD
activity
ROS
Cancer cell growth
advantage

Fig. 6


MeWo
p53
GAPDH
NT
SH1
mRNA expression (fold change)
1.2
1.0
0.8
0.6
0.4
0.2
0
NT
SH1
*
*
MnSOD
SIRT3

Suppl. Fig. 1


SIRT3 protein levels
(fold change)
1.5
1.0
0.5
0
*
siCTRL
siSIRT3
SIRT3
Tubulin
siCTRL
siSIRT3

Suppl Fig. 2

Table 1. *Primers and conditions used for RT-PCR*

gene	Forward Primer (5'- 3') Reverse Primer (5'- 3')	T An. (°C)	gene	Forward Primer (5'- 3') Reverse Primer (5'- 3')	T An. (°C)
18s	ggACACggACAggATTgACA ACCCACggAATCgAgAAAgA	60	*sod-2*	CgTgCTCCCACACATCAATC TgAACgTCACCgAggAgAAg	60
p53	ggCCCACTTCACCgTACTAA gTggTTTCAAggCCAgATgT	60	*sirt3*	CggCTCTACACgCAgAACATC CAgAggCTCCCCAAAgAACAC	56

T An.: annealing temperature; *sirt3*: sirtuin 3; *sod-2*: manganese superoxide dismutase.

Competing interests statement

All the co-authors declare that they don’t have any competing interests to declare.