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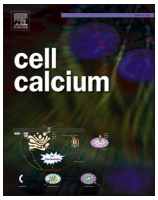

p53 alters intracellular Ca^{2+} signaling through regulation of TRPM4

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ABSTRACT

Altered expression of transient receptor potential channel melastatin 4 (TRPM4) contributes to several diseases, including cardiac conduction disorders, immune diseases, and cancer. Yet the underlying mechanisms of TRPM4 expression changes remain elusive. In this study, we report that loss of tumor suppressor protein p53 or p63γ function or mutation of a putative p53 response element in the TRPM4 promoter region increase TRPM4 promoter activity in the colorectal cancer cell line HCT 116. In cells that lack p53 expression, we observed increased TRPM4 mRNA and protein levels and TRPM4-mediated Na^+ currents. This phenotype can be reversed by transient overexpression of p53. In the prostate cancer cell line LNCaP, which expresses p53 endogenously, p53 overexpression decreases TRPM4-mediated currents. As in other cancer cells, CRISPR-Cas9 mediated knockout of TRPM4 in p53 deficient HCT 116 cells results in increased store-operated Ca^{2+}entry. The effect of the TRPM4 knockout is mimicked by p53 mediated suppression of TRPM4 in the parental cell line expressing TRPM4. In addition, a TRPM4 knockout-mediated shift in cell cycle is abolished upon loss of p53. Taken together, these findings indicate that p53 represses TRPM4 expression, thereby altering cellular Ca^{2+} signaling and that TRPM4 adds to cell cycle shift dependent on p53 signaling. One sentence summary: TRPM4 is repressed in the p53 pathway leading to reduced currents and increased calcium signaling.

Introduction

In 2020, an estimated 1.9 million cases of colorectal cancer (CRC) and 900.000 CRC-related fatalities were registered according to the world health organization [1]. Although cancer is a multifactorial disease, most cancers share common mutations in certain regulatory genes [2]. One of those genes is the tumor suppressor gene *TP53*. As a transcription factor p53, the gene product of *TP53*, binds to a multitude of target genes and initiates transcription [3]. Amongst those are genes that regulate cell cycle arrest, e.g. *CDKN1A* ($p21^{WAF1/CIP1}$), *RPRM* (Reprimo), and *SNF* (14-3-3σ), or apoptosis, e.g *PIDD1* and *BAX* [4]. Yet, less is known about genes that do not directly cause cell cycle arrest or apoptosis, including genes which encode ion channels [5,6].

Multiple pathological events can lead to loss of p53 functionality. The most common events, with 80% across all cancer types, are missense mutations at a few hotspot amino acids within the DNA-binding domain of p53, e.g. p53 R175H, hindering transactivational function [7–9]. Although a lot of research is focused on loss of transactivational function of p53, some studies also report p53-mediated repression of target genes [10–14].

Canonical p53 binding sites consist of two palindromic half sites with the sequence motif RRR-CWWG-YYY (R= purine, W = adenine or thymine, Y = pyrimidine) that are spaced by 0 to 21 base pairs [15]. In a recent study, Tebaldi *et al.* developed a pattern search algorithm to identify putative p53 response elements (RE). Several of those REs were detected upstream of the transient receptor potential melastatin 4 (TRPM4) channel gene [16]. p53 family members p63 and p73 share binding sites with p53 but also have binding sites that are exclusive to p63 and p73 [17–19]. In addition, p63 and p73 can act as collaborative partners or competitors to p53 [20].

TRPM4 is a Ca^{2+}-activated non-selective monovalent cation channel mainly permeable to Na^+ and K^+ but impermeable to Ca^{2+} [21,22]. The channel is expressed in various tissues and has several physiological functions in the immune system [23] and in the heart [24–26]. TRPM4 is a negative feedback regulator of intracellular Ca^{2+} signaling, as Na^+ influx via TRPM4 reduces the driving force for Ca^{2+} into the cell [21,27].

Ca^{2+} mediates a multitude of cellular processes including gene

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expression, neurotransmitter release, cell proliferation and apoptosis. In non-excitable cells, Ca^{2+} influx is mostly driven by Ca^{2+}-selective Orai channels [28]. Upon endoplasmic reticulum (ER) Ca^{2+} depletion, STIM1 (Stromal Interaction Molecule 1) Ca^{2+} sensors in the ER membrane activate Orai channels in the plasma membrane, and the resulting Ca^{2+} influx is called store-operated Ca^{2+} entry (SOCE).

Besides the negative feedback regulation of SOCE [21,23,29–34], TRPM4 was shown to interact with different proteins including proteins in the adhesome [35–37]. In cancer, expression of TRPM4 is upregulated and overexpression contributes to cancer hallmark function such as increased adhesion, migration, proliferation, and invasion in prostate cancer [36,38–41] and in breast and cervical cancer cells [42–45]. In addition, our group could show that TRPM4 is highly upregulated in tumor buds, a characteristic of epithelial-mesenchymal transition, and that TRPM4 contributes to proliferation, invasion, and a shift in cell cycle in colorectal cancer cells [46,47]. Yet, the underlying mechanism of TRPM4 upregulation in cancer remains elusive.

Given p53's role as a transcription factor, we aimed to elucidate if expression or loss of p53 alters TRPM4 expression and function in HCT 116 cells. In addition, we investigated if loss of p53 altered SOCE and the TRPM4-mediated cell cycle shift.

2. Material & methods

2.1. Cell culture

Cells have been cultured at 37°C and 5 % CO_2 in McCoy's 5A medium supplemented with 10 % FCS.

2.2. Luciferase assay

In a 24-well plate 70'000 HCT 116 cells were plated and 24 h after transfected in triplicates with 400 ng TRPM4 Promoter-FLuc, 50 ng pGL4.70[hRLuc] and 25 ng pcDNA3.1 empty vector or overexpressing p53, p63α, p63γ, p73α, p73β, WT or MUT, respectively (X-treme GENE™ 9 DNA Transfection Reagent, Roche, XTG9-RO). 24 h later, Firefly and Renilla luciferase units were measured using Dual-Luciferase Reporter Assay™ (Promega, E1910) and GloMax® 20-20 luminometer. Cells were washed with PBS and lysed with 100 µL 1x PLB for 15 min. 20 µL lysate was added to 100 µL LARII reagent and Firefly luciferase activity was recorded after 10 s measurement period. After addition of 100 µL Stop&Glo reagent, Renilla luciferase activity was documented.

2.3. Transfection

1 µg of DNA was transfected into 1 × 10^6 cells with the 4D-Nucleofector™ (Lonza) and SE Cell Line 4D-Nucleofector™ X Kit L (Lonza, V4XC-1012) according to the manufacturer's guidelines.

2.4. Electrophysiology

All patch-clamp experiments were performed at room temperature in whole-cell configuration. 50 ms voltage ramps from -100 to 100 mV were applied from a holding potential of 0 mV every 2 s over a time of 430 s. Currents were acquired with a EPC-10 amplifier, digitized, and recorded with PATCHMASTER v2 × 53 (both HEKA). Voltages were corrected for a 10-mV liquid junction potential. Currents were filtered at 1 kHz and then sampled at 3 kHz. Currents were extracted at −80 and 80 mV, normalized to cell capacitance and plotted versus time. Data were analyzed with IGOR PRO 6.37 and GraphPad Prism 9. Bath solutions contained: 140 mM NaCl, 0.5 mM $CaCl_2$, 3 mM $MgCl_2$, and 10 mM HEPES. In the *N*-methyl-D-glucamine (NMDG) bath solution, 140 mM NaCl was replaced by 140 mM NMDG. pH was adjusted to 7.2 with NaOH or HCl. Osmolarity was adjusted to ~ 300 mOsm with glucose. Internal solution contained 140 mM Cs-glutamate, 10 mM EDTA, 10 mM HEPES, and 8 mM NaCl. Free concentrations of 3 mM $MgCl_2$ and 500 nM $CaCl_2$ were calculated according to WEBMAXC STANDARD [48].

2.5. Western blot

Cells were lysed with mammalian protein extraction reagent (M-PER™, Thermo Fisher, 78501) and total protein content was determined with a BCA assay. 50 µg total protein were separated on a 10 % SDS PAGE gel and blotted to a nitrocellulose membrane. Proteins were probed with rabbit TRPM4 antibody (dilution 1:500; generated by Pineda [49]) and mouse β Actin antibody (dilution 1:2000; Cell Signaling Technology, #3700). Fluorescent secondary antibodies IRDye® Donkey anti-Mouse (LI-COR, #925-68022) and IRDye® 800CW Goat anti-Rabbit (LI-COR, #925-32211) were used to detect proteins in a Licor Odyssey Imaging system. Expression of TRPM4 was normalized to the expression of β Actin or γ-Tubulin (SFig. 1A-B).

2.6. Quantitative real-time PCR

QIAshredder and RNeasy Mini kit were used to isolate total RNA from HCT 116 cells. For reverse transcription, 2 µg RNA were used in the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, 4368814). After 1:4 dilution, cDNA was used for qPCR with the TaqMan Gene Expression Assay (Thermo Fisher). The following PCR conditions were used: 2-min activation at 50°C, then 10 min at 95°C; followed by 40 cycles of 15-s denaturation at 95°C and 1-min annealing at 60°C. TRPM4 expression levels were normalized to TATA box-binding protein expression.

2.7. Ca^{2+} Imaging

After transfection, cells were plated onto 25 mm glass cover slips and kept in the incubator for 48 hours. Cells were loaded with FURA-2AM (Thermo Fisher, F1221) for 15 minutes at 37°C and 5 % CO2. Prior to the measurement start cover slips were transferred into custom-built measurement chambers. After initial wash with 0.5 mM Ca^{2+}-Ringer solution, cells were allowed to equilibrate for 5 minutes before the measurement start. During the measurement, solutions were changed according to the application scheme in the relevant figures. Imaging solutions contained: 155 mM NaCl, 4.5 mM KCl, 2 mM $MgCl_2$, 10 mM D-glucose, and 5 mM HEPES. Ca^{2+} was adjusted as indicated. 0 mM Ca^{2+} was reached by addition of 1 mM EGTA. To trigger passive store depletion, 1 µM Thapsigargin (Tg, Thermo Fisher, T7459) was used.

2.8. FACS

5 × 10^5 cells have been seeded into 6-well plates and collected by scraping the 24 hours past seeding. Cells were then fixed with 70% cold ethanol and kept at 4°C for a maximum of 2 weeks. After washing with PBS cells were stained with FxCycle™ PI/RNAse (Thermo Fisher, F10797) staining solution according to the manufacturer's guidelines. PI staining was detected in a LSR II SORP flow cytometer with FACSDiva™ software (both BD Biosciences). Cell cycle distribution was analyzed with FlowJo 10.

2.9. CRISPR-Cas9 knockout

To knockout TRPM4, a deletion was generated between exons 2 and 4 (9744 bp, chromosome 19: 95 975–105 719). Two guide RNAs were designed with E-CRISP [50]: gRNA4.1/gRNA4. Sense and antisense oligonucleotides for the gRNAs were cloned into a pSpCas9(BB)-2A-GFP vector (Addgene, #48138)) or a pU6-(BbsI)-CBh-Cas9-T2A-mCherry vector (Addgene, #64324). Cloning was performed as previously described [51]. 2 × 10^6 HCT116 $p53^{-/-}$ cells were transfected with 2 µg of each gRNA with the 4D Nucleofector system (Lonza) following the manufacturer's instructions. At 24 h post-transfection, cells were sorted for GFP/mCherry with ASTRIOS flow cytometry sorter (Beckman

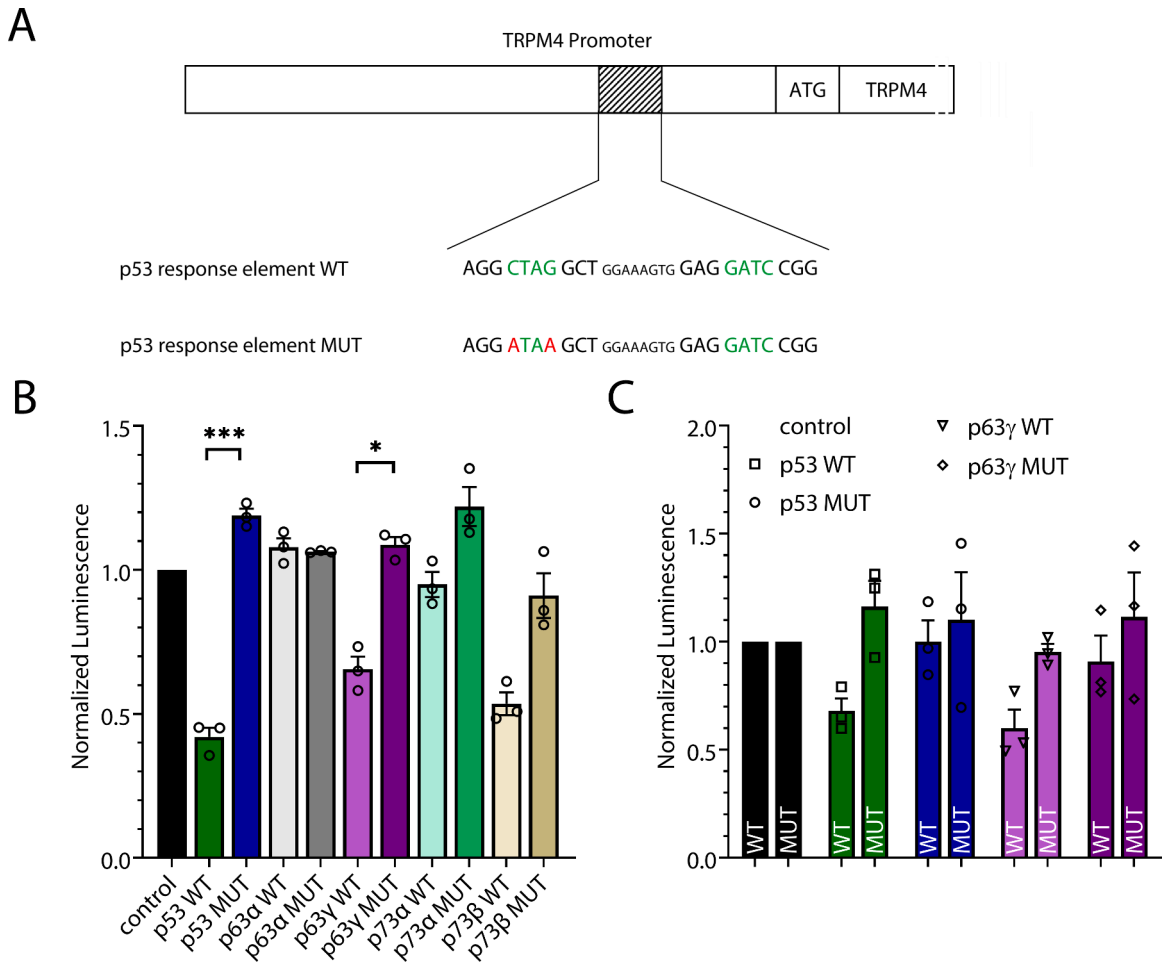

Fig. 1. TRPM4 promoter region and dual luciferase reporter assays. (A) Simplified depiction of the TRPM4 reporter region with the sequence of the wildtype p53 RE (WT) and the mutated RE (MUT). CWWG core motifs (W = adenine or thymine) are marked in green (C = Cytosine, G = Guanine, A = Adenine, T = Thymine). Spacer base pairs are in smaller font size. Mutated base pairs in red. (B) Dual luciferase reporter assay in HCT 116 cells after co-transfection of TRPM4 promoter luciferase reporter plasmid with expression plasmids of p53 family members (p53, p63α, p63γ, p73α, and p73β), corresponding non-DNA-binding mutants (p53 R175H, p63 α+γ R304H, p73 α+β R293H) or empty vector control (N = 3 experiments). Luciferase-activity has been normalized to the control. (C) Same experimental setting as in (B) with p53 and p63γ, the corresponding non-DNA-binding mutants and additional expression of the mutated (MUT) TRPM4 promoter region. Luciferase-activity has been normalized to the corresponding control (N = 3 experiments). Statistical differences were evaluated with a Brown-Forsythe and Welch ANOVA test.

Coulter). Clones were screened by genotyping. For sequences for guide RNAs and primers for genotyping please see our previous work [46].

2.10. Constructs

For the luciferase assay, the human TRPM4 promoter region, ranging from -448 to +21 bp relative to the start codon, was cloned into a pGL4.10[luc2] vector. Mutation in the putative p53 binding region was performed by GenScript.

3. Results

3.1. p53 RE mediates a reduction in TRPM4 promoter activity

We checked the TRPM4 promoter region for potential p53 RE and identified a sequence that might function as p53 RE (Fig. 1A). To evaluate the ability of p53 to alter TRPM4 promoter activity, dual luciferase assays have been performed. Overexpression of wild-type p53 (p53 WT) in HCT 116 cells leads to a significant reduction of TRPM4 promoter activity, while the non-DNA-binding mutant p53 R175H (p53 MUT) does not (Fig. 1B). We also checked for repression by members of the p53 protein family and found that also wild-type p63γ (p63γ WT) represses promoter activity significantly, while overexpression of wild-type p63α (p63α WT) has no effect. In addition, p73β WT also decreases TRPM4 promoter activity but to a lesser extent than p53 WT and p63γ WT. The corresponding non-DNA-binding mutants, p63 R304H and p73 R293H, do not suppress TRPM4 promotor activity. To test if the reduction in promoter activity is mediated by the potential p53 RE, we mutated two amino acids (Fig. 1A, marked in red) in the RE sequence. Mutation of this sequence abolishes p53 and p63γ mediated reduction in TRPM4 promoter activity (Fig. 1C). Taken together, overexpression of both p53 and p63γ reduce TRPM4 promoter activity significantly. This effect is impeded by mutation of the first half-site of the core CWWG motif.

3.2. HCT 116 cells deficient in p53 expression show increased Na^+ currents, elevated TRPM4 mRNA and protein expression

We used the HCT 116 parental cells and p53-deficient HCT 116 cell line (HCT 116 $p53^{-/-}$) that was originally generated by Bunz *et al* [52] and determined TRPM4-mediated currents. Currents were evoked with 500 nM Ca^{2+} in the patch pipette. As demonstrated earlier, these Ca^{2+} activated currents in HCT 116 are completely abolished upon knockout of TRPM4, identifying TRPM4 as the molecular basis [46]. TRPM4 mediated inward currents are blocked by replacement of Na^+ by the impermeable cation $NMDG^+$ in the bath solution (Fig. 2A). To analyze

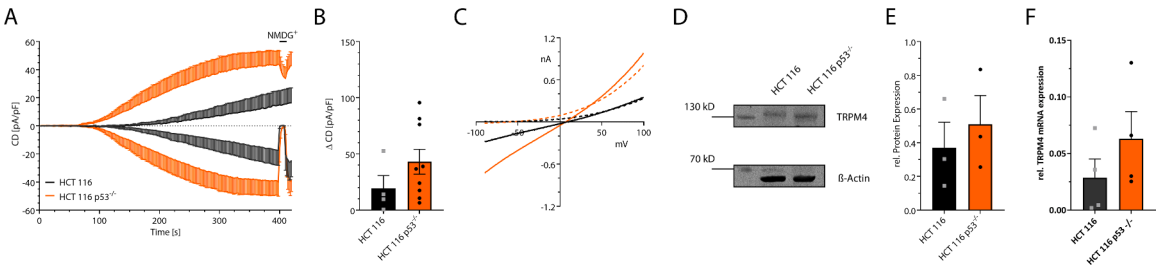

Fig. 2. Patch clamp recordings, TRPM4 protein and mRNA quantification in HCT 116 and HCT 116 p53−/−. (A) Current densities plotted over time and the corresponding current voltage relationships for HCT 116 (N = 4 cells) and for HCT 116 p53−/− (N = 9 cells). (B) Current densities extracted from (A) at t = 398 s. (C) Current-voltage relationship corresponding to (A) at t = 398 s (solid line) and t = 406 s (dotted line). (D) Representative western blot. (E) Relative TRPM4 protein expression (N = 3 experiments). (F) Relative TRPM4 mRNA expression (N = 4 experiments). Statistical differences were evaluated with one-way ANOVA.

the TRPM4 mediated inward currents in HCT 116 and HCT 116 p53$^{-/-}$, we determined the delta current density between the current in NMDG^{+} and Na^{+} (Fig. 2B). The delta current density is increased more than twofold in HCT 116 p53$^{-/-}$ versus HCT 116 with 43.0 and 19.3 pA/pF, respectively. The current-voltage relationships are linear and show positive reversal potentials in the low mV range characteristic for TRPM4-mediated currents (Fig. 2C). In addition, RT-PCR and western blot indicate increased levels of TRPM4 mRNA and protein expression when p53 is absent (Fig. 2D-F). To summarize, when p53 is absent, HCT 116 cells show increased TRPM4 currents and have increased TRPM4 mRNA and protein levels.

3.3. Overexpression of p53 WT decreases TRPM4 currents of HCT 116 cells

To test if increased TRPM4 currents are a direct effect of p53 loss, we transiently overexpressed p53 WT, p53 MUT or an empty vector as control in HCT 116 p53$^{-/-}$. p53 WT and p53 MUT were cloned into bicistronic vectors expressing GFP in parallel. We measured TRPM4 currents only in cells that were positive in GFP reporter gene expression. In cells that overexpress p53 WT, currents are reduced significantly. Overexpression of p53 MUT also reduces measured currents, but to a lesser extent than p53 WT. The delta current densities are 32.7, 7.9 and 13.9 pA/pF for the control cells, p53 WT and p53 MUT overexpression, respectively (Fig. 3B). In addition, the current-voltage relationships for control cells, and p53 WT and p53 MUT overexpressing cells show a profile that is characteristic to TRPM4-mediated Na^{+} currents (Fig. 3C). TRPM4 protein is slightly decreased when p53 WT is expressed, while expression of p53 MUT does not change TRPM4 protein expression (Fig 3D-E). Taken together, transient overexpression of p53 WT in HCT 116 p53$^{-/-}$ cells reduces TRPM4 protein levels and TRPM4-mediated Na^{+} currents. Overexpression of p53 MUT did not reduce TRPM4 protein levels and reduces TRPM4 currents to a lesser extent compared to p53 WT.

3.4. Overexpression of p53 WT decreases currents in prostate cancer cells expressing endogenous p53

To test if p53 also regulates TRPM4 expression in other cancer cell lines, we transfected p53 WT and p53 MUT into LNCaP prostate cancer cells that have wildtype *TP53* and determined TRPM4 currents. As shown in the experiments in Figure 4, overexpression of p53 WT leads to a reduction of TRPM4 mediated currents even though LNCaP cells endogenously express p53 [53]. In contrast to p53 WT, overexpression of p53 MUT does not reduce TRPM4 currents (Fig. 4A). The delta current densities (Fig. 4B) range from 7.9 pA/pF, in LNCaP transfected with p53 WT to 18.8 pA/pF and 12.8 pA/pF in p53 MUT and control transfected LNCaP, respectively. As in the HCT 116 cells, current-voltage relationships in LNCaP cells are characteristic for TRPM4 currents (Fig. 4C). In summary, overexpression of p53 WT reduces TRPM4 currents in LNCaP cells endogenously expressing p53.

3.5. Overexpression of p53 WT increases store-operated Ca^{2+} entry

We determined Ca^{2+} influx via SOCE with Fura-2AM-based Ca^{2+} imaging in a Ca^{2+} re-addition protocol (Fig. 5A-J). As in other cells [36], knockout of TRPM4 increased SOCE in HCT 116 p53$^{-/-}$ (Fig. 5A, control traces black and grey; Western blot analysis of HCT 116 p53$^{-/-}$ M4KO in Fig. S1). A thorough analysis of Ca^{2+} release from intracellular stores, and SOCE parameters Ca^{2+} influx rate, Ca^{2+} peak and Ca^{2+} plateau is given if Fig. 5B-E. TRPM4 KO significantly increased Ca^{2+} influx rate (+ 46 %), Ca^{2+} peak (+35 %) and Ca^{2+} plateau (+ 41 %) of SOCE. To assess the impact of p53 on TRPM4-mediated negative feedback regulation of SOCE, we overexpressed p53 WT and p53 MUT in HCT 116 p53$^{-/-}$. Cells overexpressing p53 WT have significantly increased SOCE key parameters, i.e. Ca^{2+} influx rate (+ 57 %), Ca^{2+} peak (+ 28 %), and Ca^{2+}plateau (+ 38 %). In contrast, overexpression of p53 MUT does not alter Ca^{2+} signaling parameters compared to HCT 116 p53$^{-/-}$. To test if the observed changes in Ca^{2+} signaling are specific to TRPM4 down-regulation within the p53 pathway, we also overexpressed p53 WT and

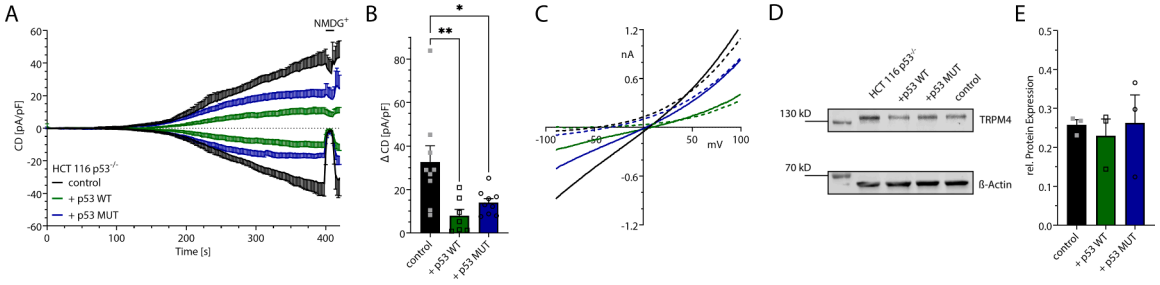

Fig. 3. Patch clamp recordings and TRPM4 protein quantification in HCT 116 p53−/−. (A) Current densities plotted over time in HCT 116 p53−/− overexpressing p53 WT (N = 7 cells), p53 MUT (N = 9 cells) or empty vector control (N = 9 cells). (B) Current densities extracted from (A) at t = 398 s. (C) Current-voltage relationship corresponding to (A) at t = 398 s (solid line) and t = 408 s (dotted line). (D) Representative western blot. (E) Relative TRPM4 protein expression (N = 3 experiments). Statistical differences were evaluated with one-way ANOVA.

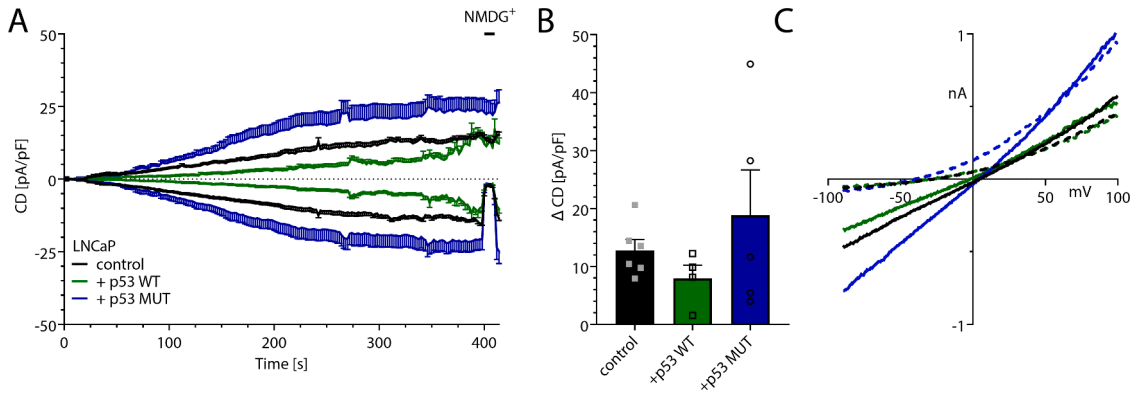

Fig. 4. Patch clamp recordings in LNCaP cells. (A) Current densities plotted over time in LNCAP cells overexpressing p53 WT (N = 4 cells), p53 MUT (N = 5 cells) or empty vector control (N = 6 cells). (B) Current densities extracted from (A) at t = 398 s. (C) Current-voltage relationship corresponding to (A) at t = 398 s (solid line) and t = 408 s (dotted line). Statistical differences were evaluated with one-way ANOVA.

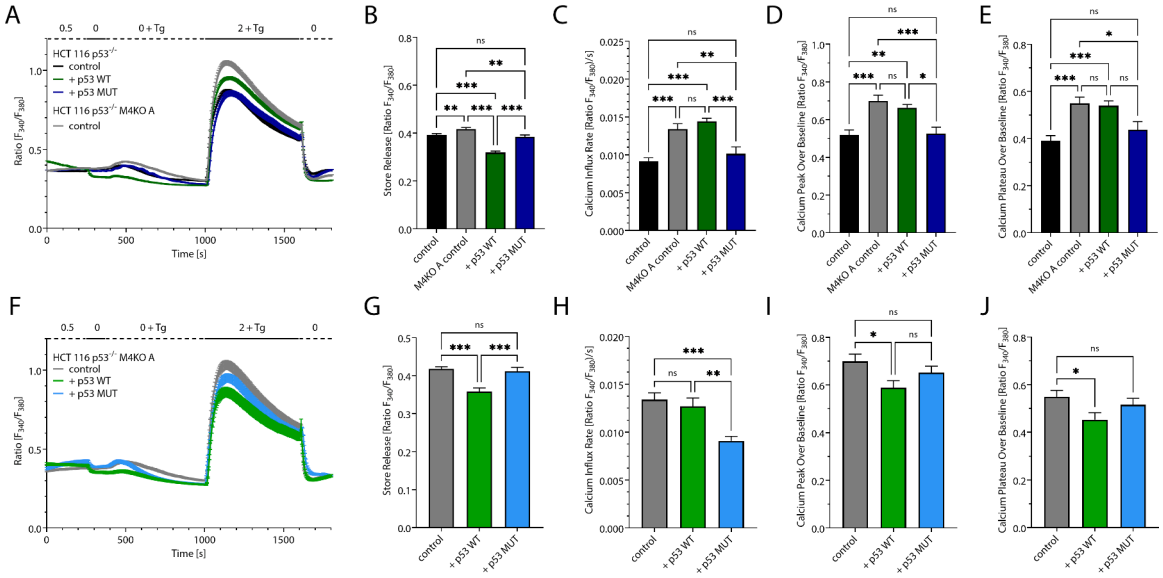

Fig. 5. Fura-2AM-based Ca^{2+} imaging in HCT 116 p53 -/- and HCT 116 p53 -/- M4KO A with overexpression of p53 WT or p53 MUT. (A) Fluorescence ratio development over time HCT 116 p53 -/- M4KO A expressing empty vector control (N = 142 cells) and in HCT 116 p53 -/- overexpressing p53 WT (N = 97 cells), p53 MUT (N = 96 cells) or empty vector control (N = 135 cells) in a standard Ca^{2+} re-addition protocol (solution changes indicated by the dotted and solid lines on top of the graph). Quantification of the store release (B), Ca^{2+} influx rate (C), Ca^{2+} peak (D), and Ca^{2+} plateau (E) from (A). (F) Same as in (A) with HCT 116 p53-/- M4KO A and overexpression of p53 WT (N = 98 cells), p53 MUT (N = 92 cells) or empty vector control (N = 142 cells, same data as in A). Quantification of the store release (G), Ca^{2+} influx rate (H), Ca^{2+} peak (I), and Ca^{2+} plateau (J) from (F). Statistical differences were evaluated with one-way ANOVA.

p53 MUT in HCT 116 $p53^{-/-}$ M4KO. In contrast, when TRPM4 is absent, overexpression of p53 WT does not increase but reduce peak Ca^{2+} and the Ca^{2+} plateau, but not Ca^{2+} influx rate. Overexpression of p53 MUT reduces Ca^{2+} influx rate, but not peak Ca^{2+} concentration and the Ca^{2+} plateau (Fig. 5F, H-J). Interestingly, independent of TRPM4 expression, overexpression of p53 WT results in reduced Ca^{2+} release from intracellular stores while store release remains unchanged when p53 MUT is expressed (Fig. 5B+G). To summarize, Ca^{2+} signaling is increased when p53 WT is overexpressed in HCT 116 $p53^{-/-}$ while overexpression of p53 MUT does not increase Ca^{2+} signaling. The increase in SOCE signaling is less pronounced compared to the increase upon knockout of TRPM4. Furthermore, the increase in Ca^{2+} signaling is specific to TRPM4 as Ca^{2+} signaling is rather slightly reduced when p53 is overexpressed in HCT 116 $p53^{-/-}$ M4KO cells.

3.6. TRPM4-mediated shift in cell cycle is dependent on p53 expression

In a previous study of our group, we could show that loss of TRPM4 expression and the pharmacological block of TRPM4 shift the cell cycle towards G1 phase [46,54]. As demonstrated earlier [46] compared to parental HCT 116, the cell cycle in two TRPM4 knockout clones (western blot analysis of TRPM4 KO in [46]) is shifted towards G1 when p53 is expressed (Fig. 6A). This shift is significant for clone M4KO 2. To test if this effect is also linked to p53 expression status we checked cell cycle distribution in HCT 116 $p53^{-/-}$ M4KO cells by FACS analysis of PI-stained cells. Compared to HCT 116 $p53^{-/-}$, none of the two M4KO clones show a shift in cell cycle (Fig. 6B). We next analyzed if the shift in cell cycle depends on p53 expression. We normalized cell cycle phases to those of the TRPM4 expressing parental cells and pooled data from all M4KO clones (HCT116 M4KO 1/M4KO 2 and HCT 116 $p53^{-/-}$ M4KO A/M4KO B). In this setting, the only difference is the p53 expression status. In this analysis, cell cycle shift is significantly increased to G1 phase, when p53 is expressed (Fig. 6C) suggesting that the TRPM4-mediated shift towards G1 phase is activated within the p53 signaling pathway.

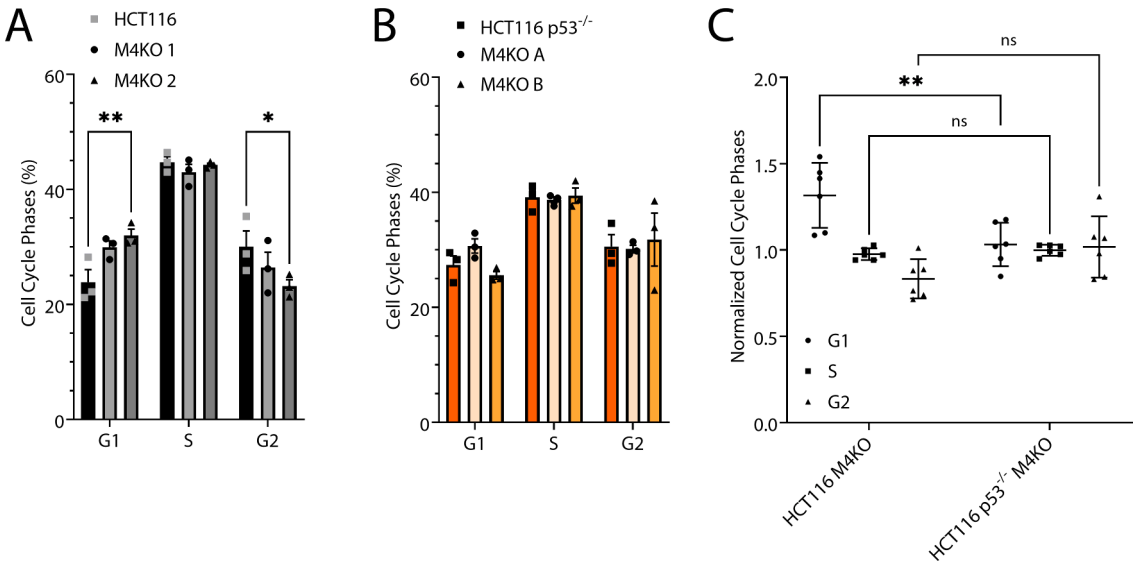

Fig. 6. FACS-based cell cycle distribution in HCT 116 and HCT 116 p53−/−. (A) Percentage of cells in G1, S, or G2 phase in HCT 116 and two clones of HCT 116 with CRISPR-Cas9-mediated TRPM4 KO (M4KO 1 and M4KO 2, N = 3 experiments). (B) Same as in (A) but with HCT 116 p53−/− (M4KO A and M4KO B). (C) Cell cycle distribution when M4KOs from (A) and (B) are pooled (two clones each) and normalized to maternal cells (N = 3 experiments). Statistical differences were evaluated with two-way ANOVA.

4. Discussion

TRPM4 has recently been considered as a cancer driver gene in PCa [55]. Further studies including our own, show that TRPM4 is upregulated in several cancer types including breast, cervical, prostate and colorectal cancer [36,43,44,56,57] and that it contributes to cancer hallmark functions [35,38–46]. The contribution of TRPM4 to cancer is well studied, yet little is known about how TRPM4 expression is regulated in physiological and pathophysiological context. Hong *et al.* report that miRNA-150 reduces TRPM4 expression and thereby suppressing epithelial-mesenchymal transition in prostate cancer [41].

Here we show that tumor suppressor protein p53 and the p53 family member p63γ reduce TRPM4 promoter activity. Mutation of the first half-site of the p53 RE abolishes p53-mediated promotor repression. Loss of p53 in turn leads to increased TRPM4-mediated Na^+ currents in HCT 116 cells. This increase in Na^+ currents can be reversed by transient overexpression of p53 in HCT 116 p53 -/- cells. Furthermore, transient overexpression of p53 also reduces Na^+ currents in LNCaP prostate cancer cells that endogenously express p53. All currents were activated with 500 nM intracellular Ca^{2+} as higher Ca^{2+} concentrations activated TRPM4-unspecific currents in p53-deficient HCT 116 cells (data not shown). p53 may regulate other ion channels e.g. Ano-1 channel that also has putative p53 REs [16] and/or in addition alter Ca^{2+} sensitivity of TRPM4. TRPM4 protein and mRNA expression are increased when p53 is absent. Transient overexpression of p53 only slightly reduces TRPM4 protein expression. In these experiments, transfected and non-transfected cells were pooled for western blot analysis which could mask the effect. When endogenous p53 is absent, an electrophoretic shift in mobility of TRPM4 is observed (Fig. 2D). This shift might be caused by p53-mediated alterations in expression of protein modifying enzymes as this has been shown for AMP-activated protein kinase [58] and FUCA1 glycosidase [59]. Subsequently, a change in posttranslational modifications e.g. glycosylation and phosphorylation could also contribute to altered TRPM4 channel activity [36]. p53 is a key regulator of cell cycle progression and apoptosis. As a transcription factor, p53 binds DNA at two decameric repeats with the sequence 5'-RRRCWWGYYY-3' (R = A or G, W = A or T, Y = C or T) that are separated by a spacer of 0-21 basepairs [15]. p53 binds to these specific sequence motifs as a tetramer where each monomer occupies one half site [60]. The sequence we found in the promoter region of TRPM4 (Fig. 1A) is not a complete fit for a canonical p53 RE. The first half site deviates from the consensus sequence in position 8, with a G instead of a C or T. The second half site has mismatches in position 4,5,7,9, and 10. But 95 % of the validated p53 RE have consensus sequence mismatches [61], either in the CWWG core or, the flanking RRR and YYY, or in both [62]. In addition, transactivational activity of p53 was also reported from single decameric half-sites and ¾ sites [63,64]. We mutated the core CWWG motif of the first half site which abolished repressive action of p53 in the luciferase assay. We conclude that the RE found in the TRPM4 promoter region is responsible for the p53-mediated repression of TRPM4.

Peuget *et al.* summarize potential mechanisms of p53 mediated repression: the well-characterized, indirect repression mediated by the p21/DREAM machinery and the indirect repression by miRNAs. Direct gene repression by p53 can be mediated via competition for binding with other transcription factors and recruitment of chromatin-modifying and co-repressor complexes [65,66]. Example genes that are repressed by competitive binding of p53 are *BCL2* (B cell lymphoma 2), *AFP* (α-Fetoprotein), and *HBV* (hepatitis B virus) enhancer element [67–69]. In contrast, c-Myc is repressed by recruitment of chromatin modifiers [70]. Since these repressive mechanisms are based on p53-binding to target sequences in the DNA, we cannot conclude by which mechanism TRPM4 repression is mediated in our study. The mode of p53 mediated TRPM4 repression remains elusive and needs further investigation. Furthermore, TRPM4 currents can be, to some extent, reduced by overexpression of p53 MUT which might indicate an additional protein-protein interaction. Indeed, p53 interacts with a multitude of proteins, including Bcl-2 protein family members, 14-3-3 family members, and transcriptionally active proteins, summarized by Fernandez-Fernandez *et al* [71]. In addition, TRPM4 was reported to associate with many different proteins summarized in Cho *et al.* [72], including the sulfonylurea receptor 1 (Sur1) [73], TRPC3 [74]; and KCTD5 [43]. Furthermore, TRPM4 is a component of the cellular adhesome during migration [35].

Na^+ influx via TRPM4 is a negative feedback regulation mechanism of store-operated Ca^{2+} entry (SOCE) in many cell types [21,23,29–33]. Here we report increased SOCE upon KO of TRPM4 in CRC cells and increased SOCE signaling upon overexpression of p53 due to reduced TRPM4 expression. This suggests that Ca^{2+} signaling is reduced once TRPM4 expression increases. This increase might partially be due to alterations within the p53 pathway. This adds to several potential

mechanisms of p53-linked dysregulation of Ca^{2+} signaling. p53 reduces expression of Bcl-2 [75], a protein that is considered to function as a Ca^{2+} leak channel in the membrane of the ER [76]. In addition, p53 was reported to interact with the sarco-endoplasmic Ca^{2+} ATPase (SERCA) thereby potentiating Ca^{2+} uptake into the ER [77]. The observed reduction in SOCE upon overexpression of p53 in TRPM4 KO cells might be caused by other players within the SOCE pathway. One potential candidate is Orai3 as Orai3 shows increased expression upon stabilization of p53 [78] and increased Orai3 expression is reported to reduce SOCE [79–85].

In a multitude of cells, SOCE signals trigger cellular processes like proliferation, migration, and apoptosis [86]. Dysregulation of SOCE signaling has been reported in several diseases, including immunodeficiency [87], tubular aggregate myopathy [88], allergy [89], nasal polyposis [90], aging [91,92] and cancer [93,94]. Particularly in cancer, studies point towards critical changes within the SOCE signaling pathway. Altered expression or function of the SOCE components Orai1 or STIM1 has been reported in several cancer tissues, including breast [95,96], cervical [97,98], colorectal [99,100], ovarian [101], prostate cancer [81,102,103], and melanoma [104–106]. Concerning Orai3 expression in cancer, reports diverge. While high Orai3 expression is reported to be a good prognostic factor in bladder cancer [107], high Orai3 expression is considered to contribute tumorigenesis [108], migration [109], and resistance to chemotherapy [110] in breast cancer. A comprehensive overview on Orai3 in cancer is given in Tanwar *et al.* [111].

Here we report an additional mechanism of altered SOCE signaling independent of the STIM/Orai machinery potentially contributing to cancer hallmark functions. SOCE signaling in colorectal cancer cells is increased by overexpression of p53 WT due to reduced TRPM4 expression, while loss of p53 function or absence of p53 increase TRPM4 mediated currents and decreases SOCE signaling. In addition, TRPM4 affects the cell cycle in the presence of p53 only and may further enhance cancer hallmark functions once p53 function is lost in late-stage colorectal cancer.

Conclusion

We show that a target sequence within the TRPM4 promoter region is responsible for p53-mediated repression of promoter activity. p53-deficient HCT 116 cells show increased TRPM4 mRNA and protein levels, increased TRPM4-mediated Na^{+} currents and decreased Ca^{2+} signaling. In addition, the cell cycle shift towards G1 phase that was observed upon knockout of TRPM4 is dependent on p53 expression. Changes in TRPM4 expression and/or in TRPM4-mediated alterations in Ca^{2+} signaling might therefore contribute to cancer hallmark functions in later cancer stages, when p53 function is lost.

Taken together our data reveal TRPM4 as a target of p53-mediated repression with consequences to Ca^{2+} homeostasis once p53-mediated transcriptional control is unleashed.

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Credit Author Statement

Sven Kappel: Conceptualization, Supervision, Project Administration, Methodology, Investigation, Formal Analysis, Visualization, Validation, Data Curation, Writing- Original Draft Preparation, **Daniela Ross-Kaschitza**: Investigation, Writing- Reviewing and Editing, **Barbara Hauert**: Investigation, **Karen Rother**: Conceptualization, Writing-Reviewing and Editing, **Christine Peinelt**: Supervision, Conceptualization, Methodology, Data curation, Writing- Reviewing and Editing, Project Administration, Resources, Funding Acquisition

Declarations of Competing Interests

none.

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