**Cell Death &
Differentiation**

ARTICLE

ΔNp63α down-regulates c-Myc modulator MM1 via E3 ligase HERC3 in the regulation of cell senescence

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Abstract

p63 and c-Myc are key transcription factors controlling genes involved in the cell cycle and cellular senescence. We previously reported that p63α can destabilize MM1 protein to derepress c-Myc, resulting in cell cycle progress and tumorigenesis. However, how the proteasomal degradation of MM1 is facilitated remains unclear. In the present study, we identified a novel E3 ligase, HERC3, which can mediate ubiquitination of MM1 and promote its proteasome-dependent degradation. We found that ΔNp63α transcriptionally up-regulates HERC3 and knockdown of HERC3 abrogates ΔNp63α-induced down-regulation of MM1. Either overexpression of MM1 or ablation of HERC3 induces cell senescence, while knockdown of MM1 rescues cell senescence induced by deficiency of either ΔNp63α or HERC3, implicating the involvement of the ΔNp63α/HERC3/MM1/c-Myc axis in the modulation of cell senescence. Additionally, our Oncomine analysis indicates activation of the ΔNp63α/HERC3/MM1/c-Myc axis in invasive breast carcinoma. Together, our data illuminate a novel axis regulating cell senescence: ΔNp63α stimulates transcription of E3 ligase HERC3, which mediates ubiquitination of c-Myc modulator MM1 and targets it to proteasomal degradation; subsequently, c-Myc is derepressed by ΔNp63α, thereby cell senescence is modulated by this axis. Our work provides a new interpretation of crosstalk between p63 and c-Myc, and also sheds new light on ΔNp63α-controlled cell senescence and tumorigenesis.

Introduction

The p63 gene, a member of the p53 family, encodes a variety of protein products, which are transcription factors playing key roles in cell proliferation, cell survival, and

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tumorigenesis. Among these isoforms, ΔNp63α is the predominant one expressed in somatic cells, especially in the epithelial basal layer in embryonic ectoderm and ectoderm-derived tissues or organs [1–7]. Owing to the lack of a full transactivation (TA) domain at their N termini, ΔN isoforms of p63, including ΔNp63α, were previously assumed to be merely antagonists of p53 as well as TA isoforms of p63 and p73 [1, 8.]. However, mounting evidence demonstrates that ΔNp63s can also stimulate a vast body of target genes involved in cell proliferation, survival, differentiation, and motility [9–12]. Like TAp63α, ΔNp63α contains a unique C terminus comprised of a sterile alpha motif and a trans-inhibitory domain, which mediates protein interaction and transactivity regulation, conferring p63α some functions distinct from those of other isoforms [1, 7, 13].

Another important transcription factor involved in the cell cycle and tumorigenesis is c-Myc, which controls transcription of a large number of genes in various animals [14–16]. In our previous study, we reported that p63α can promote proteasome-dependent degradation of c-Myc modulator MM1, resulting in derepression of c-Myc and the cell cycle [17]. However, the underlying mechanism

remains obscure. In the present study, we identified HERC3 (HECT and RLD containing E3 ubiquitin protein ligase 3) as a novel E3 ligase which mediates ubiquitination of MM1 and promotes its proteasomal degradation. Further investigation revealed that ΔNp63α transactivates HERC3, and knockdown of HERC3 abrogates ΔNp63α-induced down-regulation of MM1 protein levels. We found that either overexpression of MM1 or knockdown of HERC3 induces cell senescence, which is an important process in preventing cancer development and promoting organismal aging. Intriguingly, knockdown of MM1 can significantly rescue cell senescence induced by ablation of either ΔNp63α or HERC3. Oncomine analysis demonstrates that the ΔNp63α/HERC3/MM1/c-Myc pathway is activated in invasive breast carcinoma. These data suggest a crucial role of the ΔNp63α/HERC3/MM1/c-Myc axis in the ΔNp63α-mediated modulation of cell senescence and tumorigenesis.

Materials and methods

Cell culture, drug treatment, and MTS assay

H1299, HEK293T, and FaDu cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Hyclone). MCF-10A cells were maintained in DMEM/F12 (Hyclone), supplemented with 20 ng/ml epidermal growth factor (Invitrogen), 100 ng/ml cholera toxin (Sigma), 10 ng/ml insulin (Sigma), 500 ng/ml hydrocortisone (Sigma), 1% penicillin/streptomycin (Hyclone), and 5% horse serum (Hyclone). All cells were cultured at 37 °C in a humidified 5% CO_2 incubator. MG132 and cycloheximide (CHX) were purchased from Sigma. The MTS (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to measure cell growth and obtain a growth curve, as previously described [18].

Cell transfections and immunoblot analysis (IB)

Plasmids of pCMV-Flag-MM1, pLVX-MM1, pcDNA-ΔNp63α, and pcDNA-ΔNp63α(C306R) have been described previously [9, 17]. Myc-tagged HERC3 (Wt HERC3) and its C1018A mutant (Mut HERC3) in pCMV vectors were obtained from Dr. Hochrainer [19]. H1299 or HEK293T cells with 50% confluency in 6-well plates were transfected with 1 μg of indicated plasmids with 3 μl Lipo-Plus (Beijing Sage Creation Science Co, LTD). Then, 24 h post transfection, cells were collected, washed with phosphate-buffered saline, and resuspended in EBC250 lysis buffer (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.5% Nonidet P-40, 0.2% phenylmethylsulfonyl fluoride (PMSF),

2 μg/ml leupeptin, 2 μg/ml aprotinin, 50 mM NaF, and 0.5 mM Na_3VO_4). Protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad). An equal amount of protein (about 50 μg total protein) was loaded into each lane, separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membrane (Bio-Rad), and hybridized to an appropriate primary antibody and horseradish peroxidase-conjugated secondary antibody for subsequent detection with ECL (Millipore). Immunoblot analysis was performed with anti-MM1 (Abcam, ab129116), anti-HERC3 (Abcam, ab56440), anti-p63 (Cell Signaling Technology, cst-13109), anti-CDK4 (Santa Cruz Biotechnology, sc-260), anti-PAI-1 (Cell Signaling Technology, cst-11907), anti-c-Myc (Epitomotis, 1472-1), anti-Myc-tag (Santa Cruz Biotechnology, sc-40), anti-Flag (Abcam, ab49763), anti-Ubiquitin (Cell Signaling Technology, cst-3936), anti-GAPDH (Hua-An Biotechnology, R1208-3), and anti-Actin (Cell Signaling Technology, cst-1615).

Immunoprecipitation (IP) and ubiquitination assays

For the co-immunoprecipitation (Co-IP) experiment, H1299 cells were co-transfected to express Myc-tagged HERC3 or (and) Flag-tagged MM1. Cells were lysed in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.8, 125 mM NaCl, 5 mM $MgCl_2$, 0.25% Nonidet P-40, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM dithiothreitol) for 30 min on ice. Lysates were cleared by centrifugation at 13,000 rpm for 20 min. Protein concentration was determined using the Bradford protein assay reagent (Bio-Rad). Then, 2 mg of total proteins from cell lysates were incubated with anti-Myc-tag or anti-Flag using normal mouse IgG as control at 4 °C for 8 h, and the immune complexes were precipitated with protein G-agarose at 4 °C for 2 h. The immunoprecipitates were washed with lysis buffer, separated by SDS-PAGE, and subjected to IB for p63 and MM1.

For ubiquitination analysis, H1299 cells were transfected with pCMV-Flag-MM1 and pcDNA3-ubiquitin, plus pCMV-Myc-HERC3, pLKO.1-shHERC3 or a corresponding vector control. Then, at 48 h post transfection, cell lysates were prepared and subjected to IP using M2 anti-Flag-conjugated beads (Sigma) as described in the manufacturer's instructions. Equivalent immunoprecipitates were subjected to IB with indicated antibodies.

Reverse transcription-quantitative PCR (RT-qPCR) analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen), followed by reverse transcription using the One-Step

RT-PCR kit (Qiagen) according to the manufacturers' instructions. Quantitative PCR (qPCR) was performed using the QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer's instructions. Primer sequences were as follows: HERC3, forward 5'-TGTTGGGGATATTGGTCTCTGG-3' and reverse 5'-CCC TTG GTG TTC AAA CC ACAT-3'; p63, forward 5'-GTT ATC CGC GCC ATG CCT GTC TAC-3' and reverse 5'-TCC CCT CTA CTC GAA TCA AAT G-3'; GAPDH, forward 5'-AAA ATG GCA GTG CGT TTA G-3' and reverse 5'-TTT GAA GGC AGT CTG TCG TA-3'. Control amplification was performed using RNA samples not subjected to the reverse transcription, in parallel, to ensure no contaminating genomic DNA was present.

Lentiviral infection

An ShRNA library for human E3 ligases, including HERC3, was purchased from Open Biosystems (RHS4896). Lentivirus-based plasmids of pLVX-MM1, pLVX-ΔNp63α, pLKO.1-shp63, pLKO.1-shMM1, and pLKO.1-shGFP have been described previously [17]. The 293T cells were co-transfected with lentivirus-based plasmids along with psPAX2 and pMD2.G lentiviral packaging plasmids by LipoPlus. The media were collected and filtered through a 0.45 μM filter to remove debris 48 h later. The lentiviral particles were then concentrated by ultra-centrifugation (20,000 rpm, 2 h at 4 °C), resuspended in fresh medium at 4 °C for 24 h, then supplemented with polybrene (10 μg/ml) and used to infect H1299, FaDu, and MCF-10A cells. At 48 h after infection, the cells were subjected to IB analysis or selected in growth medium supplemented with 2 μg/ml puromycin for 2 days.

SA-β-gal staining

For senescence-associated β-galactosidase (SA-β-gal) staining, H1299 or MCF-10A cells were infected with lentiviral-based MM1, shHERC3, shp63, or (and) shMM1. Then, 48 h post infection, the cells were selected in growth medium supplemented with 2 μg/ml puromycin for 48 h. The cells were then subjected to SA-β-gal staining with SA-β-Gal Staining Kit (Beyotime, C0602). The percentage of SA-β-gal-positive cells was obtained from three repeat experiments, and t-tests were employed for statistical analysis.

Protein stability assay

Cells were transiently transfected (or infected) with indicated plasmids or small hairpin RNA (shRNA) lentiviruses. At 24 h post transfection (or infection), cells were treated with CHX at a final concentration of 100 μg/ml for additional 0–5 h. Cells were collected at the indicated time points. Equal amounts of total proteins (50 μg total protein per lane) were subjected to IB analysis. Intensities of bands for MM1 or actin were quantified with Image Lab (Bio-Rad). The protein levels of MM1 were normalized with actin, and that at time point 0 h was set as 100%.

Assays for proteasome-dependent MM1 protein degradation

H1299 cells with 50% confluency in 6-well plates were co-transfected with 1 μg pCMV2-Flag-MM1 and 1 μg pcDNA3.1 or p pCMV-myc-HERC3 by LipoPlus. After 24 h, cells were treated with 20 μM MG132 or an equal amount of solvent vehicle DMSO for 6 h more. Then, cells were collected and lysed with EBC250. Equal amounts of total protein (50 μg total protein per lane) were subjected to IB for HERC3, Myc-tag, p21, MM1, and actin.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed with anti-p63 (Cell Signaling Technology, cst-13109), using an Agarose ChIP Kit (Pierce) as described in the manufacturer's instructions. ChIPed DNA was used for qPCR, using 10% total input sheared DNA as internal controls, with the following primers: site 1, forward 5'-CAG ACT CTT CTT GCT ATG C-3' and reverse 5'- GGA GGT ACG CGG CAG CCG -3' (132 bp); site 2, forward 5'- CGG CTG CCG CGT ACC TCC -3' and reverse 5'- GGT GAC CGA AAG CCT TGG -3' (165 bp); site 3, forward 5'-CAG CGT CAA CGC TCT ATG -3' and reverse 5'- GAG GCG GGG CGG CAA GGT C-3' (163 bp). The PCR products were subjected to agarose gel electrophoresis.

Results

E3 ligase HERC3 is involved in ΔNp63α-mediated down-regulation of MM1

Our published data showed that ΔNp63α promotes proteasome-dependent degradation of MM1 protein in both H1299 and MCF-10A cells [17]; however, the underlying mechanism remains unclear. Ubiquitination mediated by specific E3 ligase is the key step during proteasomal degradation. However, the E3 ligases of MM1 remain unknown. To search specific E3 ligases of MM1 and elucidate the mechanism of how it is down-regulated by ΔNp63α, we carried out screening with an shRNA library for E3 ligases. We transfected H1299 cells with pLKO.1 constructs encoding shRNAs for different E3 ligases, which are transcriptionally controlled by or bind to p63, according to previously published papers or our own unpublished data
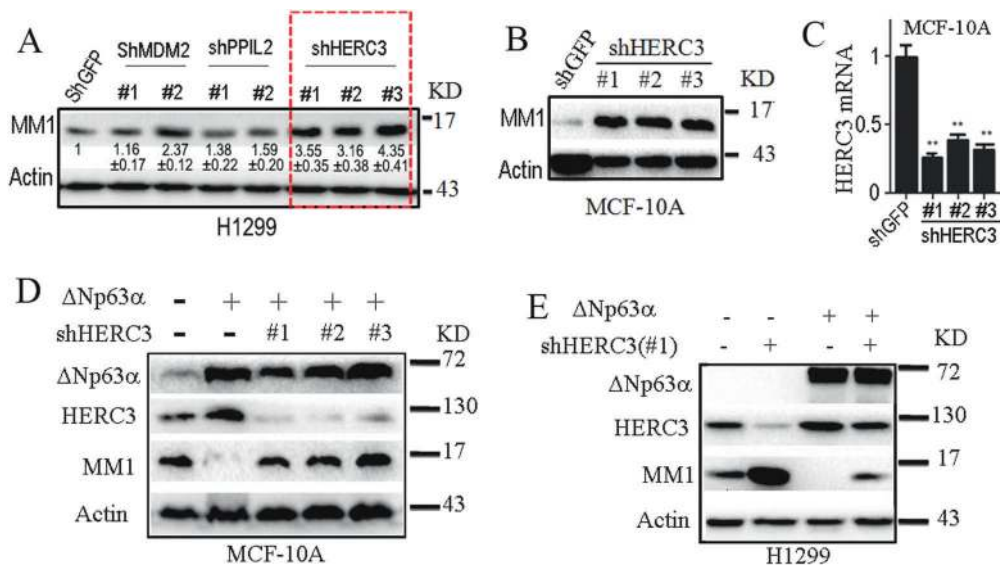
Fig. 1 Knockdown of HERC3 up-regulates MM1 and abrogates ΔNp63α-mediated down-regulation of MM1. **a–c** Knockdown of HERC3 induces up-regulation of MM1. H1299 or MCF-10A cells were transfected or infected with shRNA constructs for indicated genes from a pLKO.1 library for E3 ligases, using pLKO.1-shGFP as a control. At 48 h post transfection/infection, the cells were subjected to IB or RT-qPCR analysis. Intensities of MM1 protein bands were quantified with Image Lab (Bio-Rad, Berkeley, CA, USA) and relative intensities were normalized with actin. Quantification data of IB from two independent experiments were presented as means ± SE. The qPCR data were presented as means ± SE to test the knockdown efficiency of HERC3 (normalized with GAPDH), from three independent experiments performed in triplicate; **$p < 0.01$. **d, e** Knockdown of HERC3 abrogates ΔNp63α-mediated down-regulation of MM1. MCF-10A or H1299 cells were infected/transfected with pLVX-ΔNp63α or (and) pLKO.1-shHERC3. At 48 h post infection/transfected, the cells were subjected to IB analysis

[4, 5, 18, 20–24]. We checked 18 E3 ligases with two or three different shRNAs for each gene. Fortunately, we found that shRNA plasmids for HERC3 (shHERC3) induce a striking up-regulation of endogenous MM1 (Fig. 1a and Figure S1 in supplementary data). The shRNAs for other E3 ligases, such as MDM2 and HERC1, slightly increase the protein level of MM1, suggesting they may also contribute to proteasomal degradation of MM1 protein. To confirm the effect of HERC3 on MM1 protein levels, we prepared lentiviruses of pLKO.1-shHERC3 and infected MCF-10A cells with them. The results showed that up-regulation of MM1 induced by depletion of HERC3 also occurs in MCF-10A cells (Fig. 1b, c). Further study revealed that knockdown of HERC3 abrogates ΔNp63α-mediated down-regulation of MM1 protein levels (Fig. 1d, e). This suggests that the E3 ligase HERC3 is involved in ΔNp63α-mediated down-regulation of MM1.

HERC3 is a novel E3 ligase of MM1 mediating its ubiquitination and proteasomal degradation

Our previous study revealed that ΔNp63α shortens the half-life of MM1 protein [17]. To verify the effect of HERC3 on MM1 protein, we employed a CHX-chase assay to measure the half-life of MM1 protein in the presence or absence of ectopic expression of HERC3. The results demonstrate that, like ΔNp63α does [17], overexpression of HERC3 shortens the MM1 protein half-life (Fig. 2a, b); on the other hand, knockdown of endogenous HERC3 strikingly extends the protein half-life of MM1 (Fig. 2c, d). The Co-IP assay demonstrates that either ectopically or endogenously expressed proteins of HERC3 and MM1 form a stable complex in H1299 cells (Fig. 2e–g). These results suggest that HERC3 directly binds to MM1 protein and shortens its half-life.

HERC3 is an E3 ligase whose enzymatic activity can be removed by C1018A mutation [19]. To check whether the enzymatic activity of HERC3 is crucial to the degradation of MM1 protein, we compared the effects of wild-type (Wt HERC3) and C1018A mutant HERC3 (Mut HERC3) on MM1 protein levels. The results demonstrate that C1018A mutation almost completely abrogates HERC3 ability to down-regulate MM1 (Fig. 2h). To know if this regulation is dependent on proteasomal degradation, we treated cells with proteasome inhibitor MG132. The results demonstrate that p21, which is a well-known protein undergoing proteasomal degradation, is significantly up-regulated by MG132, indicating the effectiveness of this drug; and MG132 obviously rescues the down-regulation of MM1 mediated by HERC3 (Fig. 2i). On the other hand, the ubiquitination assay demonstrates that HERC3 dramatically increases, while knockdown of HERC3 reduces, poly-ubiquitination modification of MM1 (Fig. 2j). These data indicate that HERC3 is a novel E3 ligase of MM1, which mediates its ubiquitination and proteasomal degradation.
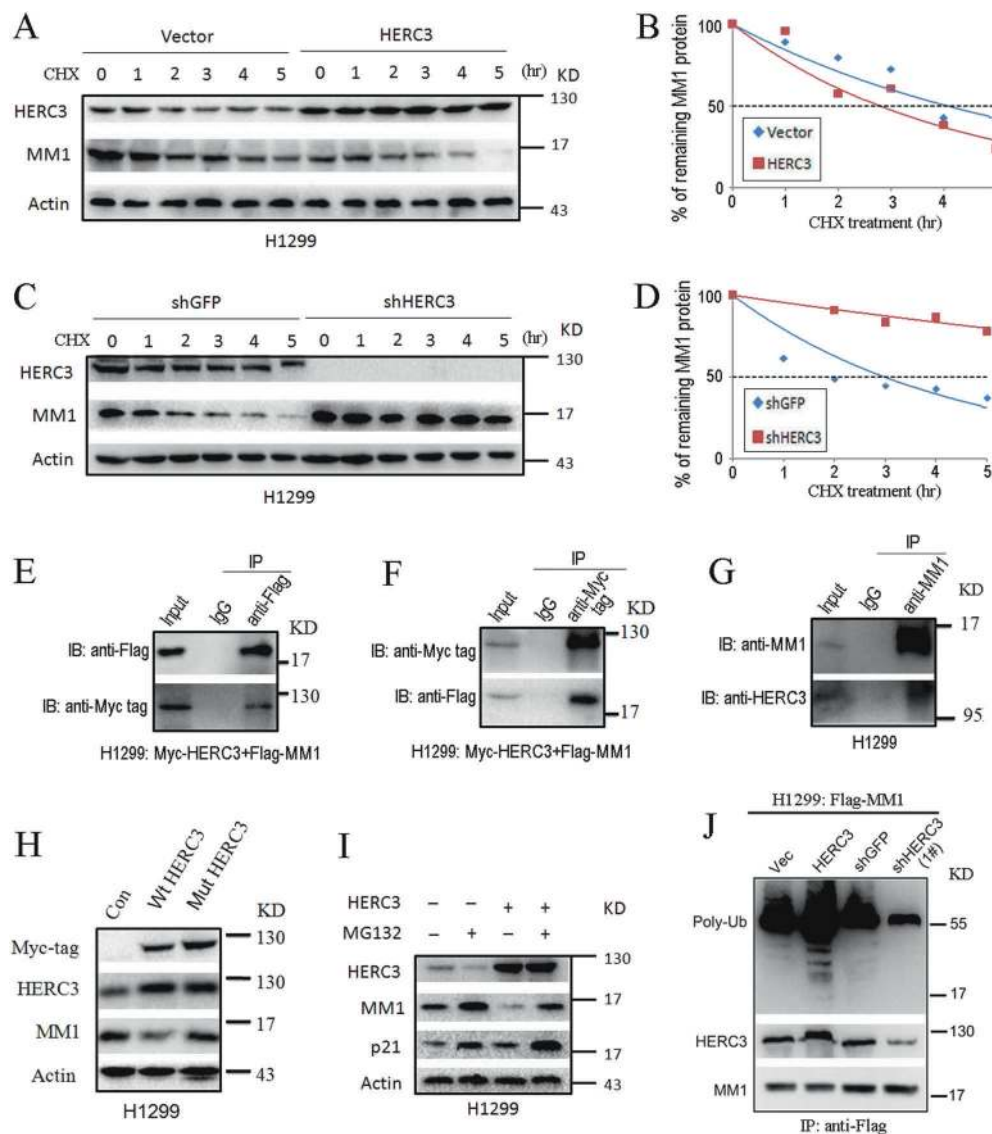
Fig. 2 HERC3 is a novel E3 ligase of MM1 mediating its ubiquitination and proteasomal degradation. **a**, **c** The half-life of MM1 protein is shortened by HERC3. H1299 cells were transfected with HERC3 or vector control. At 24 h post transfection, cells were treated with 100 µg/ml cycloheximide (CHX) for indicated time intervals, followed by IB analysis. **b**, **d** Quantification results of (**a**, **c**) respectively. Percentages of MM1 protein were normalized with actin, and the value at time point 0 h was set as 100%. **e**, **f** Exogenous HERC3 and MM1 proteins form stable complexes. Myc-tagged HERC3 and Flag-tagged MM1 were transiently overexpressed in H1299 cells. At 48 h post transfection, cells were harvested and cell lysates were subjected to IP with antibody to Flag or Myc-tag, using normal mouse IgG (IgG) as a control. **g** Endogenous HERC3 and MM1 proteins form stable complexes. Cell lysate of H1299 cells was subjected to IP with antibody to MM1, using normal mouse IgG as a control. The immunoprecipitates were analyzed by IB with indicated antibodies. Whole cell lysates were used as input controls. **h** HERC3 down-regulates MM1 dependently of its E3 ligase activity. H1299 cells were transfected with Myc-tagged wild-type HERC3 (Wt HERC3) or its C1018A mutant (Mut HERC3), which loses E3 ligase activity, using their empty vector pCMV as a control (Con). At 48 h post transfection, cells were lysed and subjected to IB analysis. **i** HERC3 down-regulates MM1 dependently of proteasome. H1299 cells were transfected with HERC3 or its vector control. At 24 h post transfection, cells were treated with 20 µM MG132 or an equal volume of solvent vehicle DMSO for additional 6 h. Cells were then harvested and subjected to IB analysis. **j** HERC3 mediates poly-ubiquitination of MM1. H1299 cells were transfected with Flag-tagged MM1 and ubiquitin, plus HERC3, shHERC3 (1#) or their vector controls. At 48 h post transfection, cells were lysed and subjected to IP with anti-Flag-conjugated beads. The immunoprecipitates were subjected to IB analysis for ubiquitinated MM1 with anti-ubiquitin or for indicated proteins

ΔNp63α transactivates HERC3 gene to increase its expression

ΔNp63α functions as a transcription factor that exerts its transactivity via binding to promoter regions of its target genes. To check whether down-regulation of MM1 induced by ΔNp63α depends on its ability to transactivate downstream genes, we used a plasmid of ΔNp63α with a C306R point mutation, which fails to bind to p63 response elements [9]. The results demonstrate that, compared to wild-type

Fig. 3 ΔNp63α transactivates HERC3 gene to increase its expression. **a** C306R mutant ΔNp63α fails to down-regulate MM1. Vector control, ΔNp63α, or ΔNp63α(C306R) plasmid was transfected into H1299 cells. After 48 h, cells were lysed and subjected to IB with indicated antibodies. **b–f** Overexpression of wild-type ΔNp63α, but not its C306R mutant, enhances HERC3 mRNA and protein levels. H1299 or MCF-10A cells were transiently overexpressed with wild-type ΔNp63α or its C306R mutant, which loses the ability to transactivate its downstream genes. At 48 h post transfection/ infection, cells were harvested and subjected to IB or RT-qPCR analysis. The qPCR data are presented as means ± SE to measure mRNA levels of HERC3 (normalized with GAPDH), from three independent experiments performed in triplicate; **$p < 0.01$. **g** Promoter region of HERC3 gene contains three potential p63 binding sites (highlighted sequences). **h** p63 directly binds to sites 1 and 3 within HERC3 promoter. Sheared genomic DNA from MCF-10A cells was subjected to ChIP assay using anti-p63 or normal mouse IgG (IgG). The immunoprecipitates or sheared genomic DNA (gDNA) were subjected to PCR amplification to detect fragments respectively containing the three sites
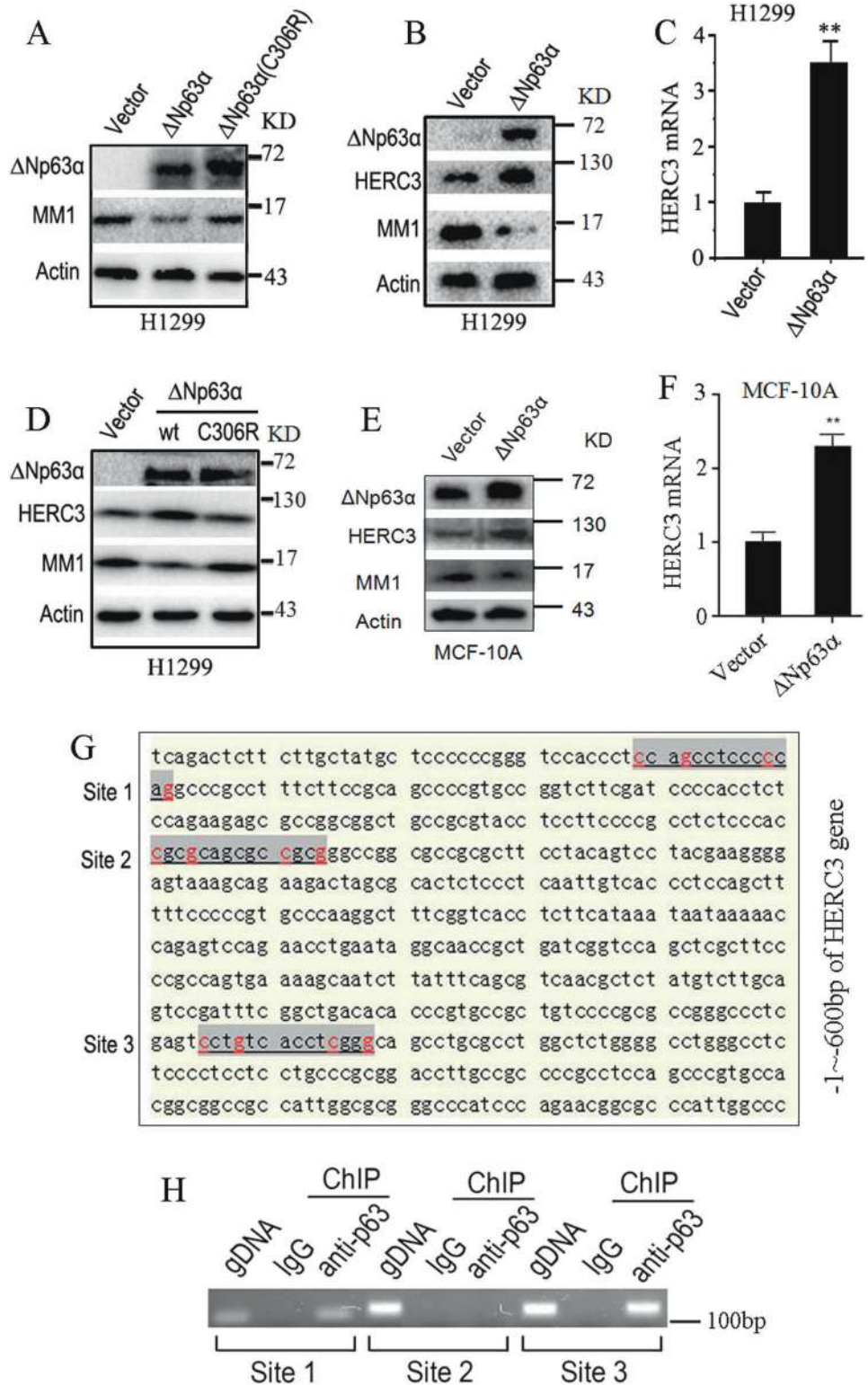

ΔNp63α, the C306R mutant fails to down-regulate MM1 (Fig. 3a). This suggests that ΔNp63α promotes MM1 degradation via regulation of certain target genes.

Since Fig. 1d, e demonstrates that HERC3 is involved in ΔNp63α-mediated down-regulation of MM1, we wondered if the expression of HERC3 can be stimulated by ΔNp63α. Our results show that overexpression of ΔNp63α in H1299 cells significantly increases expression of HERC3 (Fig. 3b). TAp63α, which was previously reported to down-regulate MM1 like ΔNp63α does, can also increase HERC3 protein

Fig. 4 Overexpression of MM1 prevents cell proliferation and induces cell senescence. MCF-10A or H1299 cells were infected/transfected with MM1 or vector control. At 48 h post infected/transfected, cells were selected with 2 μM puromycin for 2 days. The surviving cells were split into 12-well plates for MTS assay at indicated time points (**a**, **e**), SA-β-Gal staining (**b**, **c**, **f**, **g**), or IB analysis (**d**, **h**). Data are presented as means ± SE from three independent experiments performed in triplicate; $**p < 0.01$; $***p < 0.001$
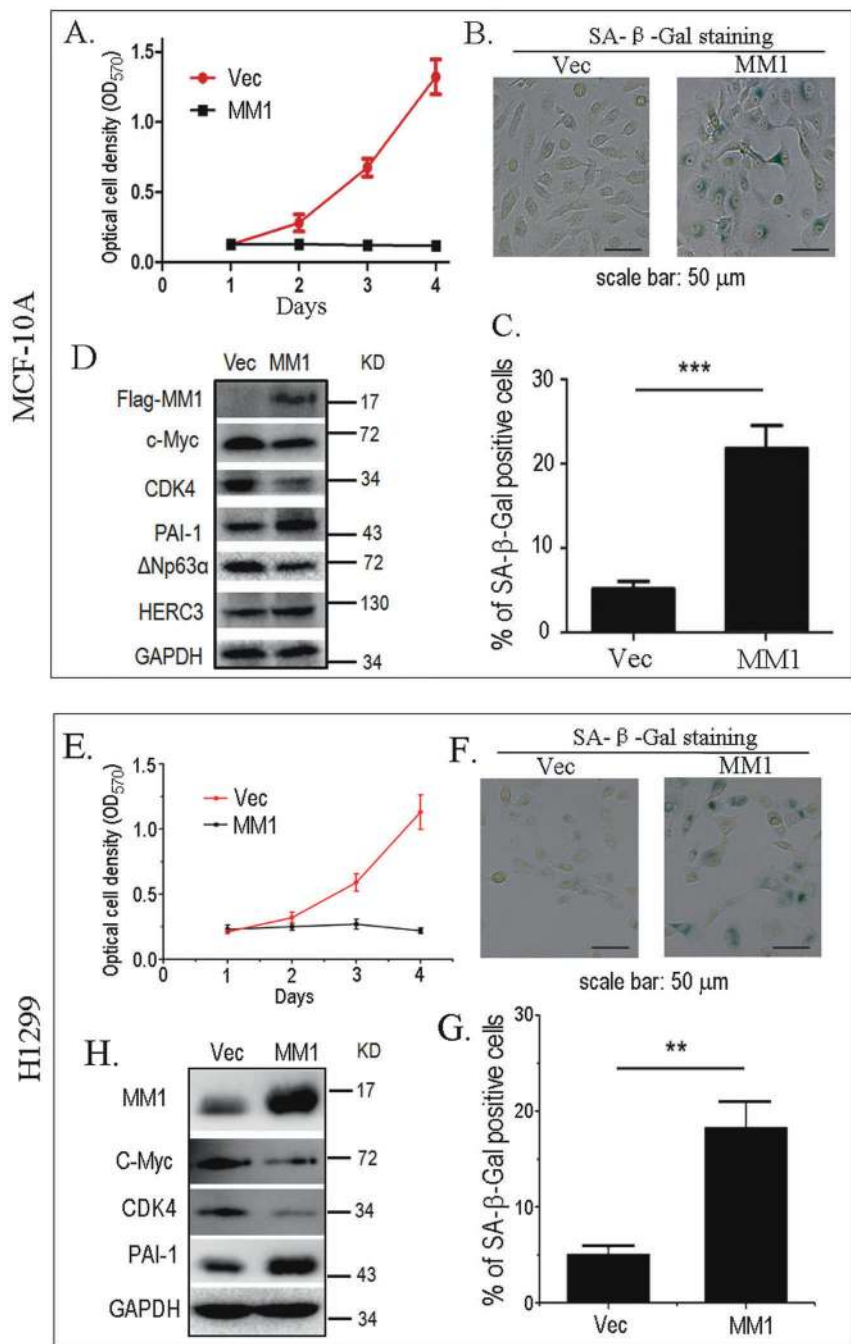
levels (Figure S2A in supplementary data). Since ΔNp63α and TAp63α are both transcription factors, we employed the RT-qPCR assay to investigate if this up-regulation of HERC3 occurs at the messenger RNA (mRNA) level. The results reveal that the HERC3 mRNA level is significantly elevated by ΔNp63α (Fig. 3c). However, the C306R mutant ΔNp63α, which loses the ability to bind to p63 response elements to transactivate the downstream genes, fails to up-regulate HERC3 (Fig. 3d). This ΔNp63α-induced expression of HERC3 also occurs in MCF-10A cells (Fig. 3e, f).

On the other hand, knockdown of p63 in MCF-10A or FaDu cells, which predominantly express ΔNp63α isoform, results in a striking down-regulation of HERC3 (Figures S2B, C in supplementary data). As a transcription factor, ΔNp63α prefers to recognize and bind to a consensus sequence CNNGNNNNNNCNNG in the promoter regions of various genes to stimulate their transcription [25]. It was reported that ΔNp63α may bind to promoter of HERC3 gene [4]. Our analysis reveals that there are three potential binding sites of ΔNp63α in the HERC3 promoter

Fig. 5 Ablation of MM1 partially rescues cell senescence induced by ΔNp63α knockdown. MCF-10A cells were infected with lentivirus-based shRNAs for GFP (as a control), p63, or (and) MM1. At 48 h post infection, cells were selected with 2 μM puromycin for 2 days. The surviving cells were subjected to SA-β-Gal staining (**a**), IB analysis (**b**), or MTS assay (**c**). Data are presented as means ± SE from three independent experiments performed in triplicate; ***$p <$ 0.001
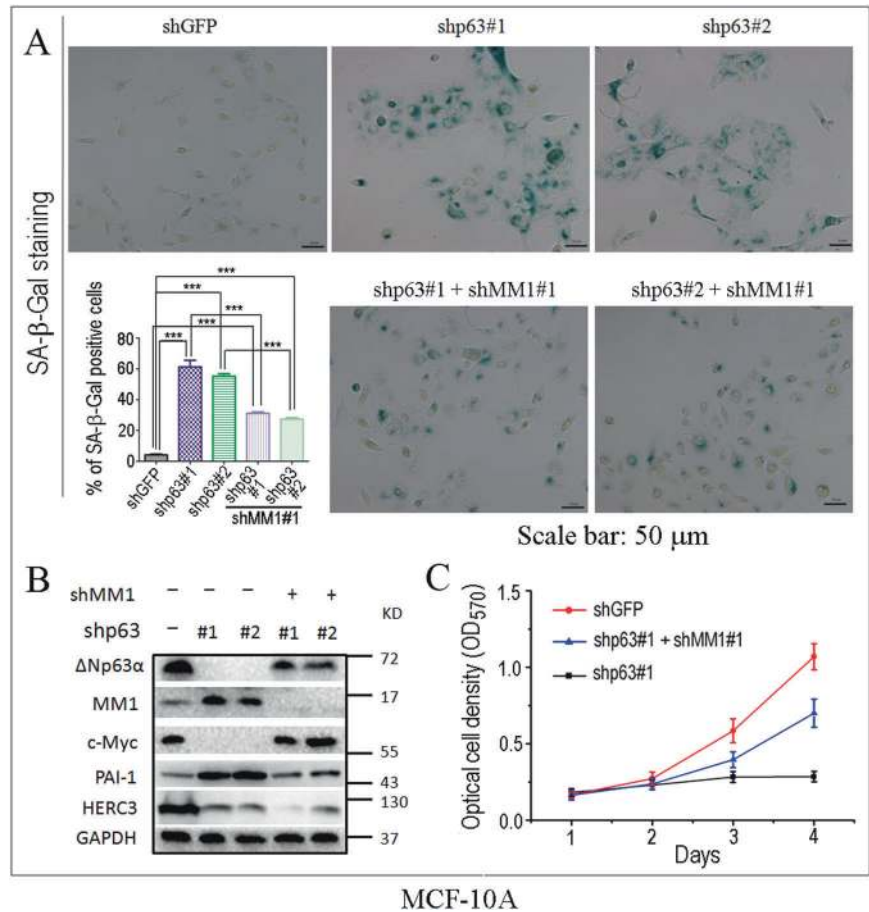
(Fig. 3g). To further validate the direct regulation of p63 on HERC3 transcription, we employed a ChIP assay. The results demonstrate that fragments containing either site 1 or site 3 can be detected in ChIPed products from sheared MCF-10A genomic DNA with anti-p63 (Fig. 3h). These results suggest that ΔNp63α transactivates the HERC3 gene to increase its expression.

MM1 induces proliferative cell senescence

In our previous study, we found that MM1 can modulate c-Myc transactivity and cell cycle progression [17]. To further investigate the biological functions of MM1, we over-expressed it in MCF-10A cells and found that cell pro-liferation is dramatically repressed (Fig. 4a). SA-β-Gal staining demonstrates that MM1 overexpression sig-nificantly increases the percentage of SA-β-Gal-positive cells, which is a hallmark of cell senescence (Fig. 4b, c). The IB analysis demonstrates that c-Myc and its down-stream target CDK4 are dramatically down-regulated by MM1, while the protein level of cellular senescence marker PAI-1 is elevated (Fig. 4d). These data suggest that MM1-mediated repression of c-Myc can inhibit cell proliferation and induce cell senescence. Intriguingly, we found that

ΔNp63α protein level was down-regulated in cells trans-fected with MM1 (Fig. 4d). This finding was reproducible in both MCF-10A and FaDu cells (Figures S3A, B in supplementary data). On the other hand, knockdown of MM1 results in up-regulation of endogenous ΔNp63α protein (Figures S3C, D). Further study demonstrated that inhibition of proteasome with MG132 fails to rescue MM1-mediated down-regulation of ΔNp63α (Figure S3E), while RT-qPCR results showed that MM1 induces a significant decrease in ΔNp63α mRNA level (Figure S3F). These data suggest that MM1 down-regulates ΔNp63α at the mRNA level.

To further investigate the effects of MM1 on cell senescence, we overexpressed MM1 in H1299 cells. As shown in Fig. 4e–h, our results reveal that, like in MCF-10A, MM1 also represses cell proliferation as well as expression of c-Myc and CDK4 in H1299 cells; the increases in SA-β-Gal and PAI-1 demonstrate MM1-induced H1299 cell senescence. Since H1299 cells do not express endogenous p63 proteins, cell senescence induced by MM1 might be, at least to some extent, independent of its negative regulation on p63. Taken together, our results show that MM1 induces proliferative cell senescence.

Fig. 6 Ablation of HERC3 induces cell senescence, which can be strikingly rescued by MM1 depletion. MCF-10A or H1299 cells were infected/transfected with shRNAs for GFP (as a control), HERC3, or (and) MM1. At 48 h post infection/transfection, cells were selected with 2 μM puromycin for 2 days. The surviving cells were subjected to SA-β-Gal staining (**a**, **b**, **d**, **e**) or IB analysis (**c**, **f**). Data are presented as means ± SE from three independent experiments performed in triplicate; ***$p <$ 0.001
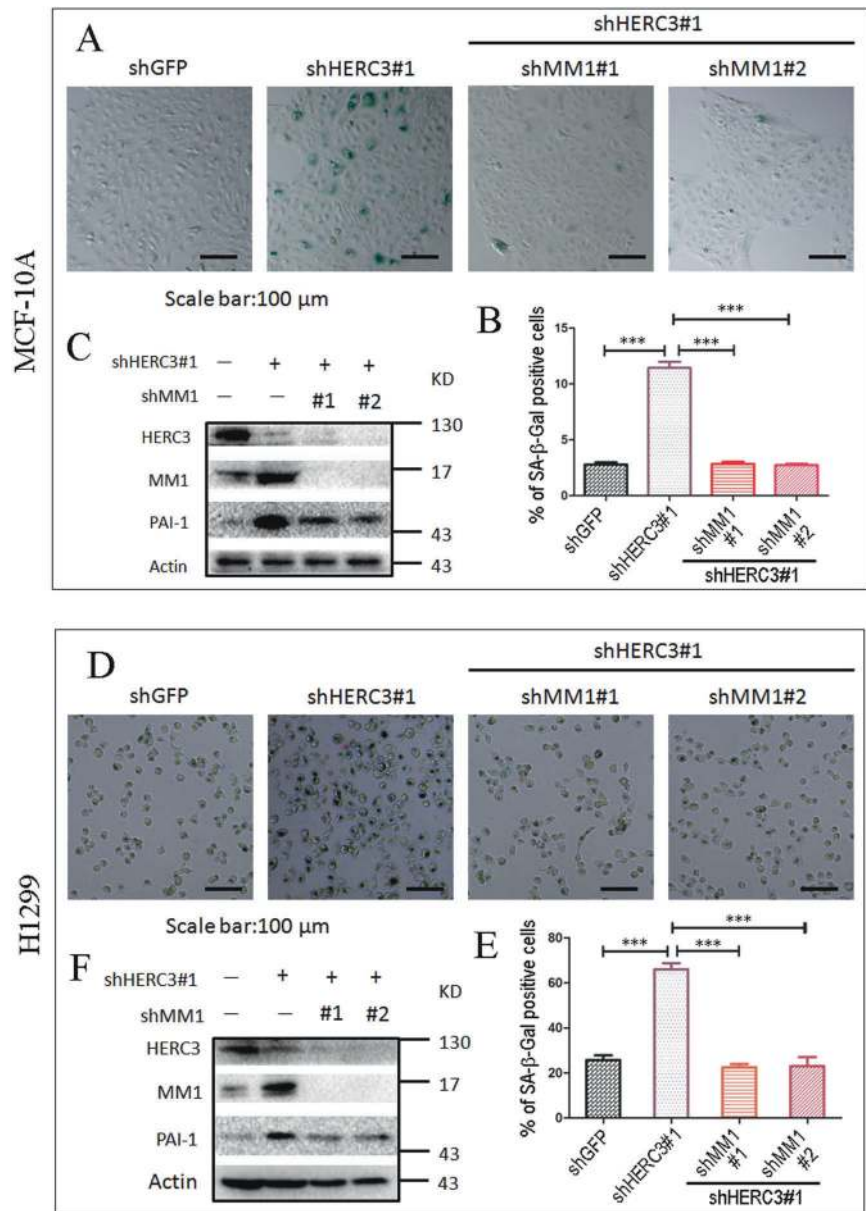
## Depletion of MM1 partially rescues cell senescence induced by ΔNp63α knockdown

It was reported that deficiency of p63, particularly its predominant isoform ΔNp63α, can trigger a cellular senescence program [26]. To check whether MM1 is involved in ΔNp63α-regulated cell senescence, we knocked down endogenous ΔNp63α and MM1 with lentivirus-based shRNAs. The results demonstrate that knockdown of ΔNp63α, which is the predominant isoform of p63 expressed in MCF-10A cells, induces a remarkable increase in the percentage of SA-β-Gal-positive cells, which can be partially, but significantly, rescued by simultaneous

knockdown of MM1 (Fig. 5a). The IB analysis confirms the alteration and restoration of both the ΔNp63α/MM1/c-Myc axis and senescence marker PAI-1 (Fig. 5b). Being consistent with the above-mentioned finding that MM1 inhibits ΔNp63α (Fig. 4d and S3), simultaneous knockdown of MM1 can restore the expression of ΔNp63α in p63-knockdown cells (Fig. 5b, lanes 4 and 5 vs lanes 2 and 3). On the other hand, ablation of ΔNp63α induces a striking repression of cell proliferation, which can be partially rescued by simultaneous knockdown of MM1 (Fig. 5c). These results suggest that elevated MM1 protein levels and subsequent repression of c-Myc, to some extent, accounts for cell senescence induced by deficiency of ΔNp63α.

Fig. 7 Oncomine analyses of p63, HERC3, MM1, and c-Myc genes in invasive breast carcinoma. Box plots represent the expression of indicated genes in invasive breast carcinoma samples (Finak Breast dataset)
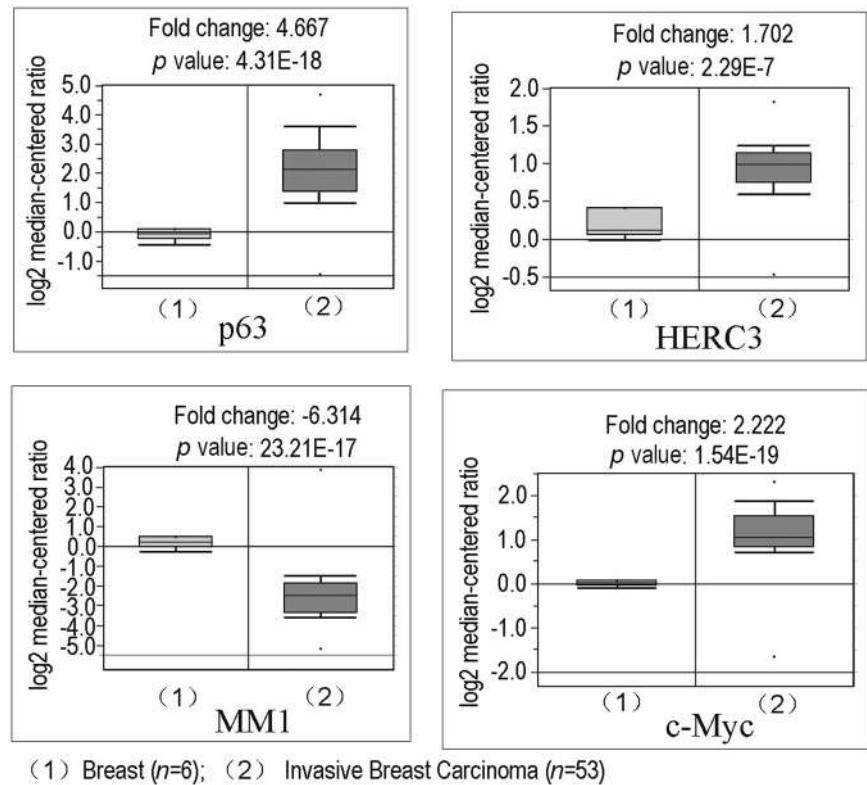
(1) Breast (*n*=6); (2) Invasive Breast Carcinoma (*n*=53)

Ablation of HERC3 induces cell senescence, which can be strikingly rescued by MM1 depletion

Since Figs. 1–3 show that HERC3 is a key regulator of the ΔNp63α/MM1/c-Myc axis, we wondered if it plays some roles in cell senescence. To this end, we knocked down HERC3 in either MCF-10A or H1299 cells and performed SA-β-Gal assays. The results demonstrate that knockdown of HERC3 with three different shRNA constructs significantly up-regulates the MM1 protein level and increases the percentage of SA-β-Gal-positive cells in either MCF-10A or H1299 cells (Figure S4). To evaluate the roles of MM1 in cell senescence induced by HERC3 knockdown, we simultaneously knocked down MM1. The results demonstrate that knockdown of MM1 can significantly rescue cell senescence induced by HERC3 ablation (Fig. 6). In combination with the abovementioned observation that MM1 represses c-Myc and induces cell senescence, these data indicate that HERC3 can modulate cell senescence via regulating MM1 protein levels.

The ΔNp63α/HERC3/MM1/c-Myc axis is up-regulated in invasive breast carcinoma

Low incidence of cell senescence is a major property of cancers, endowing them with malignant proliferation properties. Since Figs. 4–6 demonstrate that the ΔNp63α/

HERC3/MM1 axis plays a key role in the regulation of proliferative cell senescence in MCF-10A human immortalized mammary epithelial cells, we evaluated the status of this axis in breast cancers. As shown in Fig. 7, expressions of p63, HERC3, and c-Myc are significantly up-regulated in invasive breast carcinoma, while MM1 is markedly down-regulated. These results indicate that the ΔNp63α/HERC3/MM1/c-Myc axis may play some roles in the tumorigenesis of invasive breast carcinoma.

Discussion

Both p63 and c-Myc are important transcription factors playing key roles in cell cycle progress and tumorigenesis via regulating the expression of a range of downstream genes [1–3, 14–16]. It has been reported that p63 positively regulates c-Myc via Wnt/β-catenin and Notch signaling pathways, governing keratinocyte proliferation or differentiation [27]. Our previous data show that p63α promotes protein degradation of c-Myc modulator MM1, thereby depressing c-Myc and consequently regulating cell cycle progression [17]. To elucidate the underlying mechanism of how p63α facilitates proteasome-dependent degradation of MM1, we performed a screening to search for the specific E3 ubiquitin ligase targeting MM1 to proteasomal degradation. We found that HERC3 mediates poly-ubiquitination of MM1 protein and accelerates its proteasome-dependent

degradation, suggesting that HERC3 is a novel E3 ubiquitin ligase for MM1 protein. On the other hand, HERC3 knockdown abrogates ΔNp63α-induced MM1 down-regulation, suggesting it is a key mediator in this process. Additionally, we identified HERC3 as a novel downstream target gene of ΔNp63α, which positively regulates HERC3 mRNA levels. Considering the fact that we only checked a small fraction of the whole library of E3 ligases, and that shHERC3 is simply the most robust one in up-regulating MM1 protein levels, we do not rule out the possibility that there may be other E3 ligases involved in this ΔNp63α-induced MM1 down-regulation. Collectively, our data illuminate a novel ΔNp63α/HERC3/MM1/c-Myc axis: ΔNp63α transactivates E3 ligase HERC3, which mediates ubiquitination of MM1 and subsequently down-regulates it via accelerating its degradation. According to data from other groups, MM1 may negatively regulate c-Myc via either inhibiting its transactivity or facilitating its degradation [28–30].

As a modulator of c-Myc, which controls approximately 15% of genes ranging from *Drosophila* to humans and is activated in more than 20% of malignant tumors [31, 32], MM1 shows features of a tumor suppressor. The research group of Ariga [33] found that in about 50–60% of leukemia/lymphoma cells, and in more than 75% of squamous cell carcinoma of tongue cancer, MM1 is mutated and fails to repress c-Myc transactivity. However, how MM1 is regulated remains unknown. Our identification of HERC3 as a novel E3 ligase that targets MM1 to proteasomal degradation helps our understanding of the protein stability regulation of this important co-factor.

Despite the fact that HERC3 is highly homologous to other members of the HERC family and is found to associate with ubiquitin [34], no evidence so far shows that HERC3 functions as an E3 ubiquitin ligase targeting proteins to proteasome-mediated degradation [35, 36]. Hochrainer et al. [19] reported that HERC3 provides a link between nuclear factor-κB RelA and proteasome to facilitate degradation of RelA. However, this process is independent of the enzymatic activity of HERC3 [19]. Our data, for the first time, prove that HERC3 can mediate protein ubiquitination and thereby accelerate proteasome-dependent protein degradation. Additionally, we found that ΔNp63α can transcriptionally up-regulate HERC3, helping us to understand the regulation of this E3 ligase, whose mutation has been found to associate with colorectal and gastric carcinomas [37].

By halting cell proliferation, cell senescence plays key roles either against tumorigenesis or in leading to organismal aging [38, 39]. Both ΔNp63α and c-Myc are key transcription factors promoting cell proliferation and opposing cell senescence via enhancing E2F activity or maintaining telomeres through multiple pathways [15, 16, 20, 26, 40, 41]. In the present study, we show that ΔNp63α inhibits proliferative cell senescence via positively regulating c-Myc: ΔNp63α may up-regulate c-Myc via either our newly found ΔNp63α/HERC3/MM1/c-Myc axis or the previously reported Wnt/β-catenin and Notch signaling pathways [27]. The partial rescue effect of MM1 ablation on cell senescence induced by ΔNp63α deficiency suggests that the ΔNp63α/HERC3/MM1/c-Myc axis accounts for ΔNp63α regulation of cell senescence to some extent. ΔNp63α may also bypass this axis and directly modulate some cell cycle regulators to inhibit senescence. The Oncomine analysis (Fig. 7) showed that ΔNp63α, HERC3, and c-Myc are up-regulated in invasive breast carcinoma, indicating that activation of this axis may play some roles in tumorigenesis. Of note, the Oncomine analysis reveals only mRNA levels. Our hypothesis of the ΔNp63α/HERC3/MM1/c-Myc axis assumes that activation of ΔNp63α leads to up-regulation of HERC3 and c-Myc at the mRNA level, as well as down-regulation of MM1 at the protein level. Down-regulation of MM1 mRNA shown in the Oncomine results indicates that other mechanisms may be involved in the regulation of MM1 and c-Myc during tumorigenesis of invasive breast carcinoma. Additionally, the fact that MM1 negatively regulates transcription of ΔNp63α (Figure S3) suggests a feedback loop between them.

Taken together, our work elucidates a novel pathway in which ΔNp63α positively regulates c-Myc, and strongly implicates its role in cell senescence and tumorigenesis.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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