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BRD4 sustains p63 transcriptional program in keratinocytes

E. Foffi[1], A. Violante[1], R. Pecorari[2], A. M. Lena[1], F. Rugolo[1], G. Melino[1] and E. Candi[1,2*]

Abstract

Here, we investigated the potential interaction between bromodomain-containing protein 4 (BRD4), an established epigenetic modulator and transcriptional coactivator, and p63, a member of the p53 transcription factor family, essential for epithelial development and skin homeostasis. Our protein–protein interaction assays demonstrated a strong and conserved physical interaction between BRD4 and the p53 family members—p63, p73, and p53—suggesting a shared binding region among these proteins. While the role of BRD4 in cancer development through its interaction with p53 has been explored, the effects of BRD4 and Bromodomain and Extra Terminal (BET) inhibitors in non-transformed cells, such as keratinocytes, remain largely unknown. Our functional analyses revealed changes in cellular proliferation and differentiation in keratinocytes depleted of either p63 or BRD4, which were further supported by using the BRD4 inhibitor JQ1. Transcriptomic analyses, chromatin immunoprecipitation, and RT-qPCR indicated a synergistic mechanism between p63 and BRD4 in regulating the transcription of keratinocyte-specific p63 target genes, including *HK2*, *FOXM1*, and *EVPL*. This study not only highlights the complex relationship between BRD4 and p53 family members but also suggests a role for BRD4 in maintaining keratinocyte functions. Our findings pave the way for further exploration of potential therapeutic applications of BRD4 inhibitors in treating skin disorders.

Keywords Keratinocytes, BRD4, BET, P63, Proliferation, Transcription, Epigenetic regulation

Introduction

Skin keratinocytes create a tightly layered epithelium on the body's surface, serving as a protective barrier against the external environment. The formation and maintenance of the skin epidermis are regulated by dynamic, well-coordinated processes involving stemness, proliferation and differentiation [1–5].These critical cell fate decisions rely on a delicate interplay between transcription factors, chromatin dynamics, and epigenetic readers, that activate and repress specific sets of genes in a precise temporal and spatial manner [6–10]. The TP63 gene encodes multiple isoforms, of which the amino-deleted ΔNp63α isoform (here after indicated as p63) is a key regulator of epidermal development, differentiation, proliferation and self-renewal (Annie Yang, Mourad Kaghad, [2, 4, 5, 11–13]). The transcription factor p63, is a member of the p53 gene family. Unlike p53, which is well-known for its role in tumor suppression (Annie Yang, Mourad Kaghad, 2002; [14, 15]), p63 is recognized as a crucial regulator of epidermal development, as evidenced by various animal models and human diseases bearing p63 mutations [2, 5, 16–18]. For instance, mice with a complete deletion of p63 or ΔNp63 isoform, lack epidermis and related appendages and exhibit defects in other epithelial tissues [2, 5, 18, 19]. In humans, heterozygous mutations in TP63 cause several developmental disorders, many of which present with skin abnormalities [20, 21]. Several studies have established that p63 is vital for embryonic epidermal development and for the proliferation and differentiation of epidermal keratinocytes. It directly regulates numerous target genes involved in

*Correspondence:
E. Candi
candi@uniroma2.it
[1] Department of Experimental Medicine, TOR, University of Rome "Tor Vergata", 00133 Rome, Italy
[2] Istituto Dermopatico Dell'Immacolata, IDI-IRCCS, 00167 Rome, Italy

cell proliferation, differentiation, and adhesion [22–28]. Different isoforms of p63 have been identified in various cells and tissues, including the epidermis, oocytes, muscles, and cochlea [29–32]. Recent research has also shown that p63 influences the epigenetic and chromatin landscape in epidermal keratinocytes by regulating chromatin factors and by engaging and opening chromatin regions [33–39]. Studies using genome-wide approaches have confirmed that p63 is a key regulator of the enhancer landscape [34, 40], suggesting a more complex model of its role in gene regulation during epidermal development and in related diseases.

Proper timing in transcription regulation plays a crucial role in directing cell fate and influencing cell fate determination and cancer progression [41]. The Bromodomain-containing protein 4 (BRD4), a member of the BET (Bromodomain and Extra Terminal) family, facilitate the transcriptional elongation step ([42]). Notably, BRD4 binds to acetylated histones, particularly histone H4, through its two bromodomains, promoting active transcription [43]. Additionally, BRD4 is found at distal enhancer regions where its presence correlates with enhancer activity and the transcription of enhancer RNAs (eRNAs) [44–47]. Recent studies have revealed that BRD4-dependent gene expression programs are frequently disrupted in various diseases, including cancer [48]. The function of BRD4 is highly influenced by specific contexts. While several studies have highlighted BRD4's roles in aging and cancer development [49, 50], the precise mechanisms leading BRD4's actions in different normal cell types and the factors that determine its activity in various cellular contexts remain largely unknown.

In this study, we investigated the previously unknown role of BRD4 in normal human keratinocytes. We demonstrate that p63 physically interacts with BRD4, and this interaction is conserved among the p53 family members, including p53 and p73. Our experiments show that both keratinocyte proliferation and differentiation depend, at least in part, on the activities of BRD4 as further supported using the BRD4 inhibitor JQ1. Transcriptomic analyses revealed a synergistic mechanism between p63 and BRD4 in regulating p63-detendent keratinocyte-specific transcriptional program. Our findings open new avenues for developing therapeutic strategies to treat skin diseases.

Results

BRD4 is a p53 family interactor

Beyond its role as an epigenetic regulator, BRD4 also acts as a transcriptional coactivator. Therefore, BRD4 could be considered a relevant p63 and p53 family member co-binding factor. Given the high structural similarity of the p53 family members (Fig. 1a), we decided to investigate if BRD4 could be a specific interactor of the p53 family. Semi-endogenous immunoprecipitation (IP) (Fig. 1b–d) revealed that BRD4 is indeed a p53 family interactor and the interaction lies in a common shared region among the oligomerization (OD) and the DNA-binding (DBD) domain (Fig. 1a). We also found that BRD4 expression was positively and significantly correlated with p53, p63, and p73 expression in different datasets of normal skin and normal squamous epithelia (Fig. S1). Since p63 is the main actor in the regulation of skin homeostasis and showed higher correlation with BRD4 (Fig. 1e), we decided to further investigate their role in epidermal development. To better understand the nature of the interaction between BRD4 and p63, we decided to verify if BRD4 activity could be required in the binding. We performed an exogenous co-immunoprecipitation in HEK293T using the wild-type (WT) and a functionally inactive version (BD) of BRD4 (Fig. 1f) co-transfected with ΔNp63α-HA [51]. As shown in Fig. 1g, BRD4 inactivation does not affect the binding with p63. Further, we used different BRD4 deleted constructs [52] (Fig. 1f) to investigate which BRD4 functional domain could be involved in the binding process. As shown in Fig. 1h, only the isoform containing the CTM domain was able to maintain the interaction. The endogenous IP in normal human epidermal keratinocytes (HEKn) combined with a proximity ligation assay (PLA) in immortalized human keratinocytes (Ker-CT), confirmed the p63 and BRD4 interaction (Fig. 1i, l). Our data indicate that BRD4 physically interacts with the p53 family of transcription factors, specifically interacting with p63 at the endogenous level in human keratinocytes.

BRD4 and p63 regulate keratinocyte proliferation and differentiation

Given the p63 crucial role in the regulation of epithelial cell proliferation and differentiation, and the physical interaction among p63 and BRD4 in keratinocytes, we examined the cell proliferation after p63 and BRD4 depletion. P63 and BRD4 knock-down by siRNA (Fig. S2 a–d) resulted in a significant accumulation of cells in the G0/G1 phase (Fig. 2a–b), with a consequent reduction of cells in the S phase as evaluated by EdU-incorporation assay (Fig. 2c).

We markedly observed that cell proliferation rate was also reduced, both in depletion of BRD4 and p63 (Fig. 2d, Fig. S2d, e). Interestingly, we find comparable results after using JQ1, a specific BET inhibitor that can inactivate BRD4 activity (Fig. 2e–h) [48], confirming that BRD4 activity is necessary to support keratinocyte proliferation. Indeed, we observed a strong reduction of the number of cells in the S phase (Fig. 2g) and a high reduction of cell

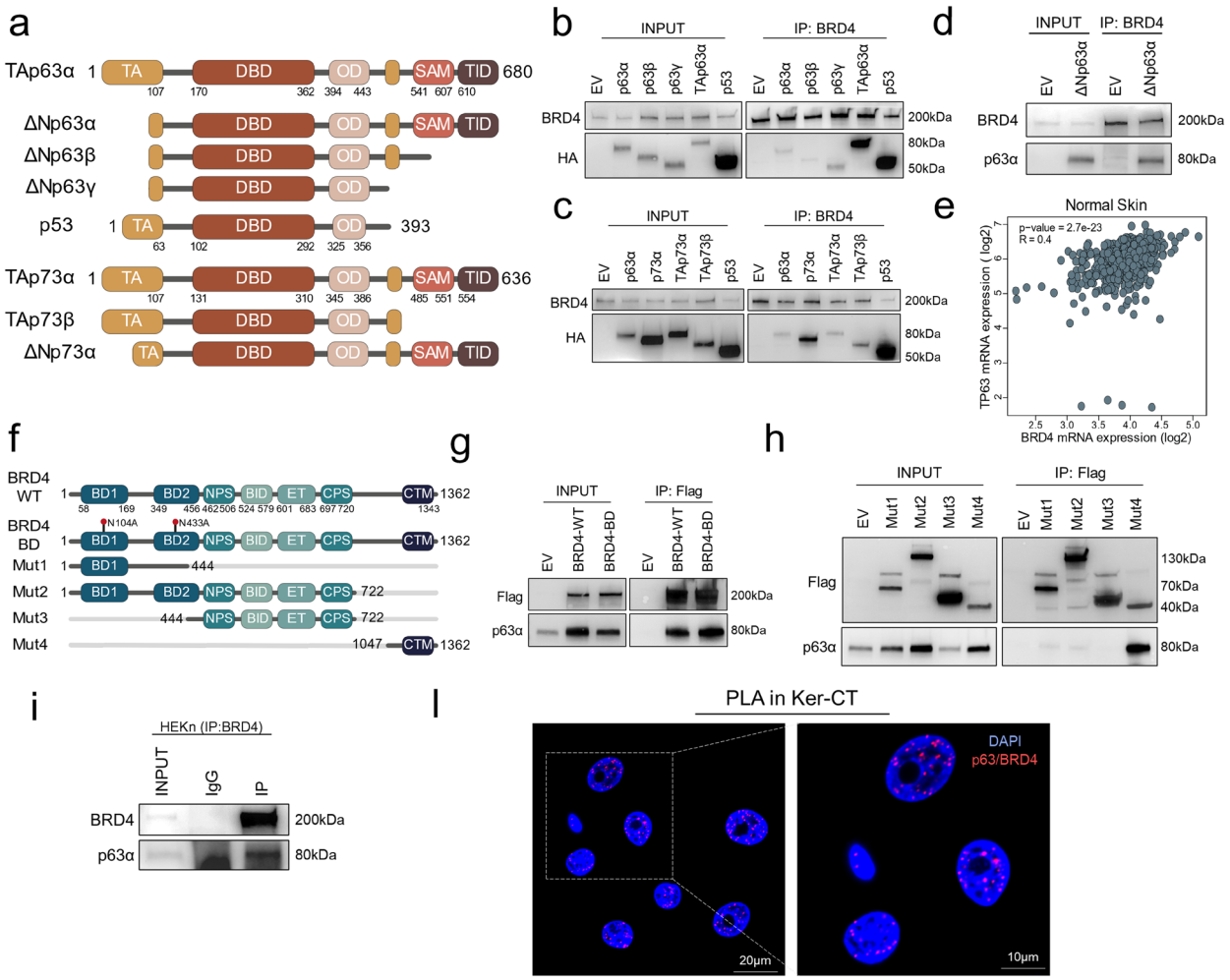

Fig. 1 BRD4 is a p53 family interactor. **a** Schematic representation of p53 family members. **b**–**d** Semi endogenous co-IP, HEK293T were transiently transfected with different isoforms of p53 family members HA-tagged. In each panel, the upper lane represents anti-BRD4 antibody, lower lane the anti-p63 antibody. EV, empty vector. Inputs are 10%. **e** BRD4 and p63 mRNA correlations in public datasets (GEPIA). **f** Schematic representation of the different BRD4 mutants. **g, h** Exogenous co-IP, HEK293T were transiently transfected with different Flag-tagged BRD4 isoforms and with HA-p63. In each panel, the upper lane represents anti-Flag antibody, lower lane the anti-p63 antibody. EV, empty vector. Inputs are 10% **i, l** Endogenous co-IP and PLA in HEKn, IP was performed utilizing anti-BRD4 antibody and analyzed with western blot: upper lane anti-BRD4 antibody, lower panel anti-p63 antibody. PLA was performed in Ker-CT with antibody recognizing p63 in combination with BRD4. The blots in the figure are representative of two independent experiments

proliferation rate (Fig. 2h). We then evaluated whether BRD4 depletion affected the expression of the early differentiation marker, keratin 10. Keratinocytes were induced to differentiate upon 1.2 mM calcium addition for 3 and 6 days in the medium upon p63 and BRD5 knockdown by siRNAs. We observed a significant decrease of keratin 10 expression both at mRNA and protein levels (Fig. 2i, l), and we found a strong and significant reduction of protein expression, particularly after BRD4 silencing (Fig. 2i, l). These findings were also confirmed after JQ1 treatment (Fig. 2m, n), in which we observed a tendence to decrease. Altogether these data demonstrate that BRD4 knock-down or inactivation affect both keratinocytes proliferation and differentiation. BRD4 down-regulation or inactivation phenocopy p63 knock-down supporting the hypothesis that BRD4 could be a positive effector of p63 transcriptional activity in keratinocytes.

BRD4 and p63 regulate the expression of selected target genes

To confirm that BRD4 participates to the p63-transcriptional program in keratinocytes, we decided to investigate three p63-target genes, *HK2*, *FOXM1* and *EVPL*. Two public datasets of ChIP-sequencing

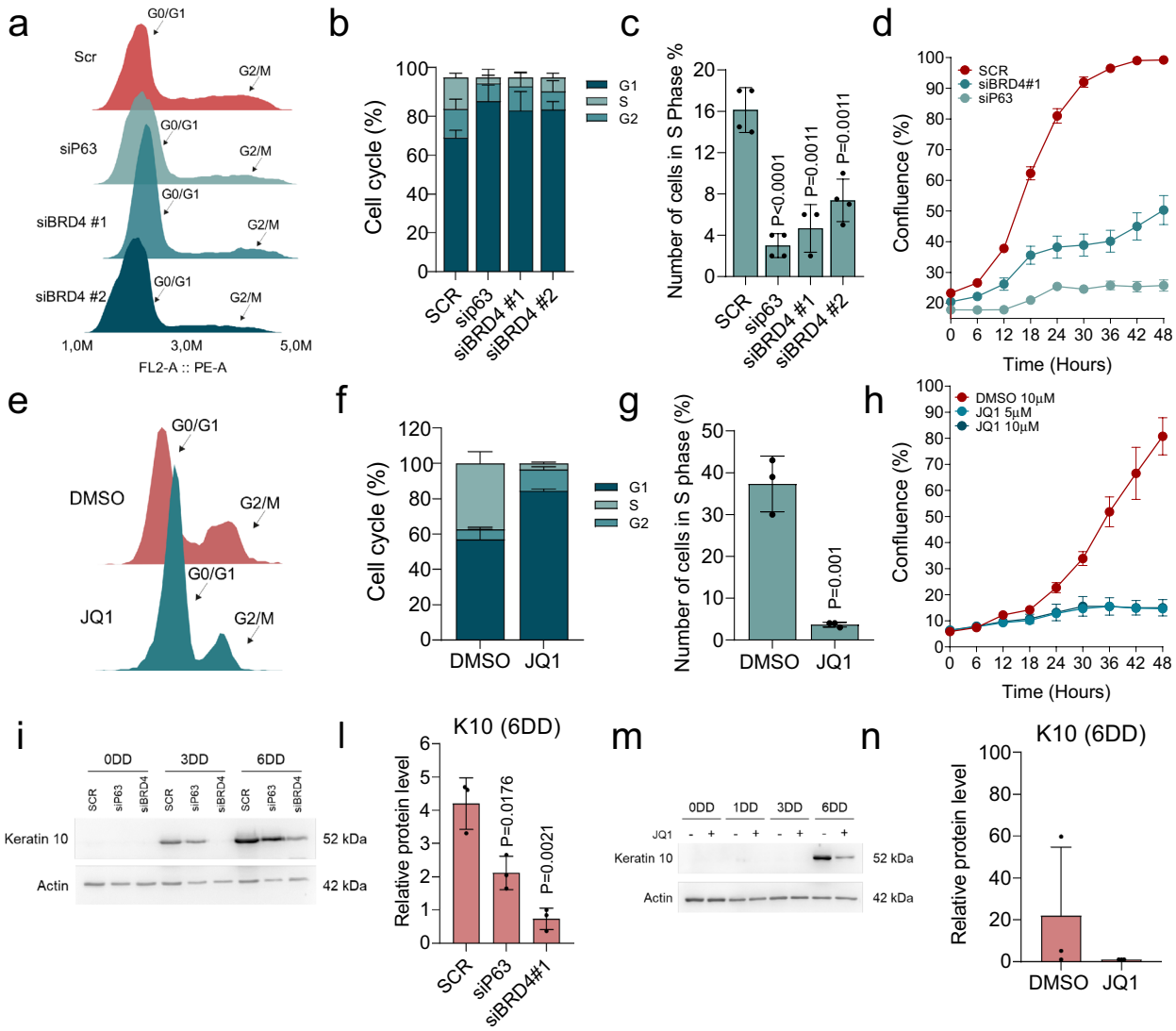

Fig. 2 BRD4 and p63 regulates keratinocytes proliferation and differentiation. **a, b, e, f** Cell cycle analysis performed in HEKn comparing SCR condition and sip63 and siBRD4 conditions comparing untreated cells (DMSO) and JQ1 treated cells (10 µM). Cells were stained with Propidium Iodide (50 µg/ml) for 1 h and then analysed by flow cytometry. In **a, e** representative plot of cell cycle analysis performed in HEKn is shown, while in **b, f** a quantification of cell cycle analysis. Graphs present means ± SD of three independent experiments. **c, g** EdU-incorporation assay of HEKn cells transfected with siSCR, sip63 and different BRD4 (siBRD4#1, BRD4#2) siRNAs or treated with DMSO and JQ1 (10 µM). Data are shown as mean ± SD of N = 3 experiments. (Unpaired Student's t test). **d, h** Growth curve of HEKn cells transfected with siSCR, sip63 and siBRD4#1, or treated with DMSO and JQ1 (10 µM). The cell confluency has been determined using the Incucyte real-time video imaging system. Each data points indicate mean ± SEM. **i–n** Western blot analysis was carried out with specific antibodies against K10, and β-actin was used as loading control. ImageJ program was used to quantitate the protein levels. The blot is representative of three independent experiments. DD, days of differentiation

analyses for p63 and BRD4 were merged with the binding profile of p63, and we identified specific binding sites (BSs) in the proximity of the *HK2*, *FOXM1* and *EVPL* promoters. The identified BRD4 and p63 BSs were overlapping, indicating that p63 and BRD4 are associated on chromatin (Fig. 3a–c). The p63 chromatin binding site were canonical sites, located at the already characterized BS for each specific gene (25, 47, 48) (Fig. 3a–c). ChIP experiments confirmed p63 and BRD4 binding (Fig. 3d–f), in which we observed an enrichment for p63 and BRD4 respectively over these regions. We also found that *HK2*, *FOMX1* and *EVPL* transcription and protein levels are reduced after the depletion of p63 and BRD4 (Fig. 3g–i, Fig. 3 j–l, Supplementary Fig. 5 a–d), indicating that their expression could be regulated by both p63 and BRD4. *HK2*, *FOMX1* and

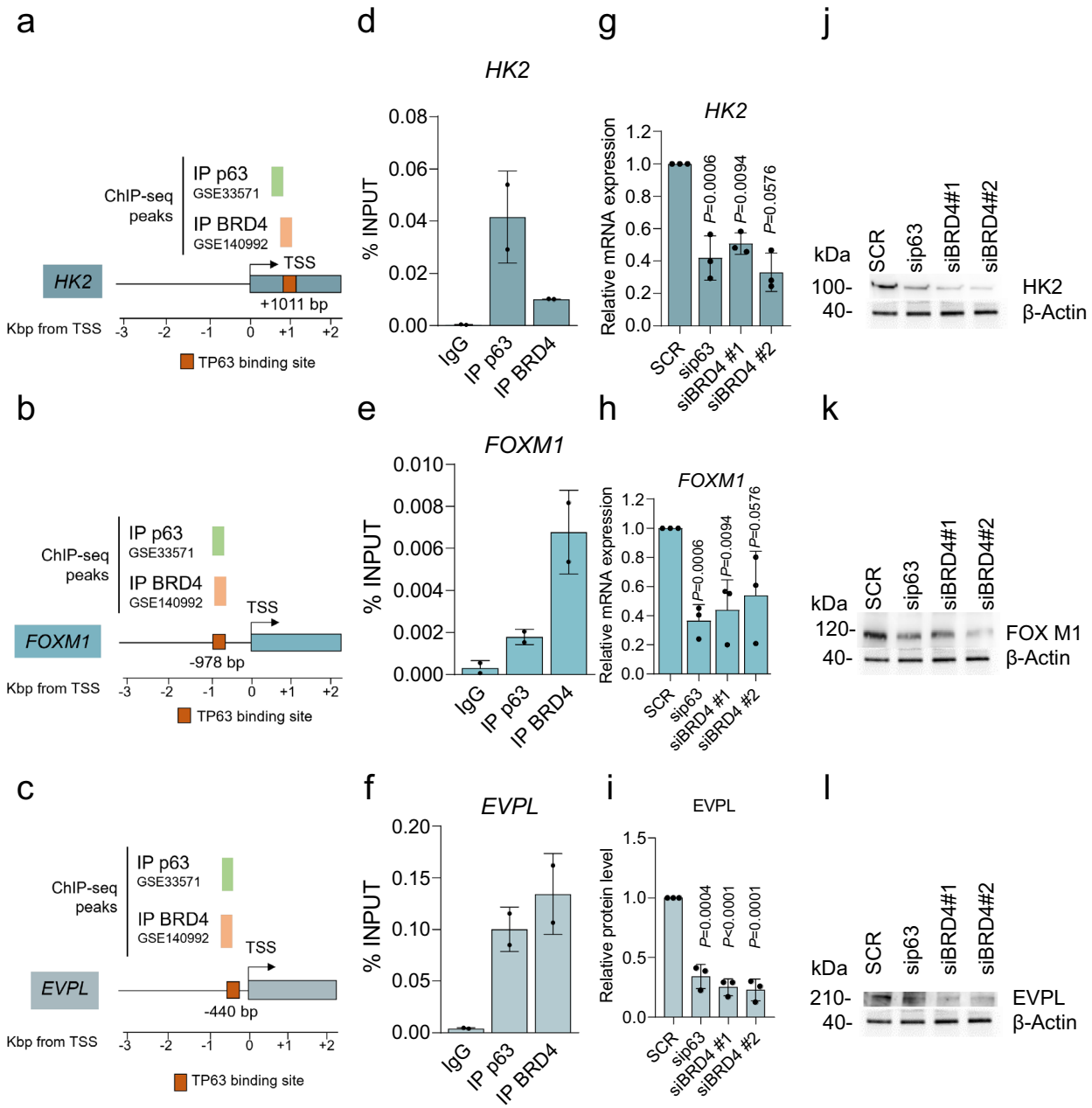

Fig. 3 BRD4 and p63 regulate the expression of selected target genes. **a**–**c** Schematic representation of the regulatory regions of the human *HK2*, *FOXM1* and *EVPL* genes in which putative p63 binding sites are highlighted. ChIP-Seq analysis using GSE140992 and GSE33571 datasets revealed the presence of BRD4 and p63 bound to human *HK2*, *FOXM1* and *EVPL* genes. TSS: transcription start site. The scheme was drawn using JASPAR (http://jaspar.genereg.net/). **d**–**f** ChIP analyses were carried out with specific antibodies for BRD4 and p63. Data are shown as mean ± SD of two independent experiments (unpaired Student's t test). **g**–**i** HEKn cells were silenced with siSCR, sip63 and two different siRNA for BRD4 for 48 h. Then RT-qPCR analyses were carried out to evaluate the mRNA levels of *HK2*, *FOXM1* and *EVPL*. Graphs present means ± SD of three independent experiments (unpaired Student's t test). **j**–**l)** HEKn cells were silenced with siSCR, sip63 and two different siRNA for BRD4 for 48 h. Then Western Blot analysis were carried out to evaluate the protein levels of *HK2*, *FOXM1* and *EVPL*. The blot is representative of three independent experiments

EVPL transcription is also reduced after JQ1 treatment (Fig. S4), indicating that loss of BRD4 activity affects the expression of selected p63 target genes. These data together suggest that BRD4 and p63 cooperate in the transcriptional regulation of *HK2*, *FOXM1* and *EVLP*.

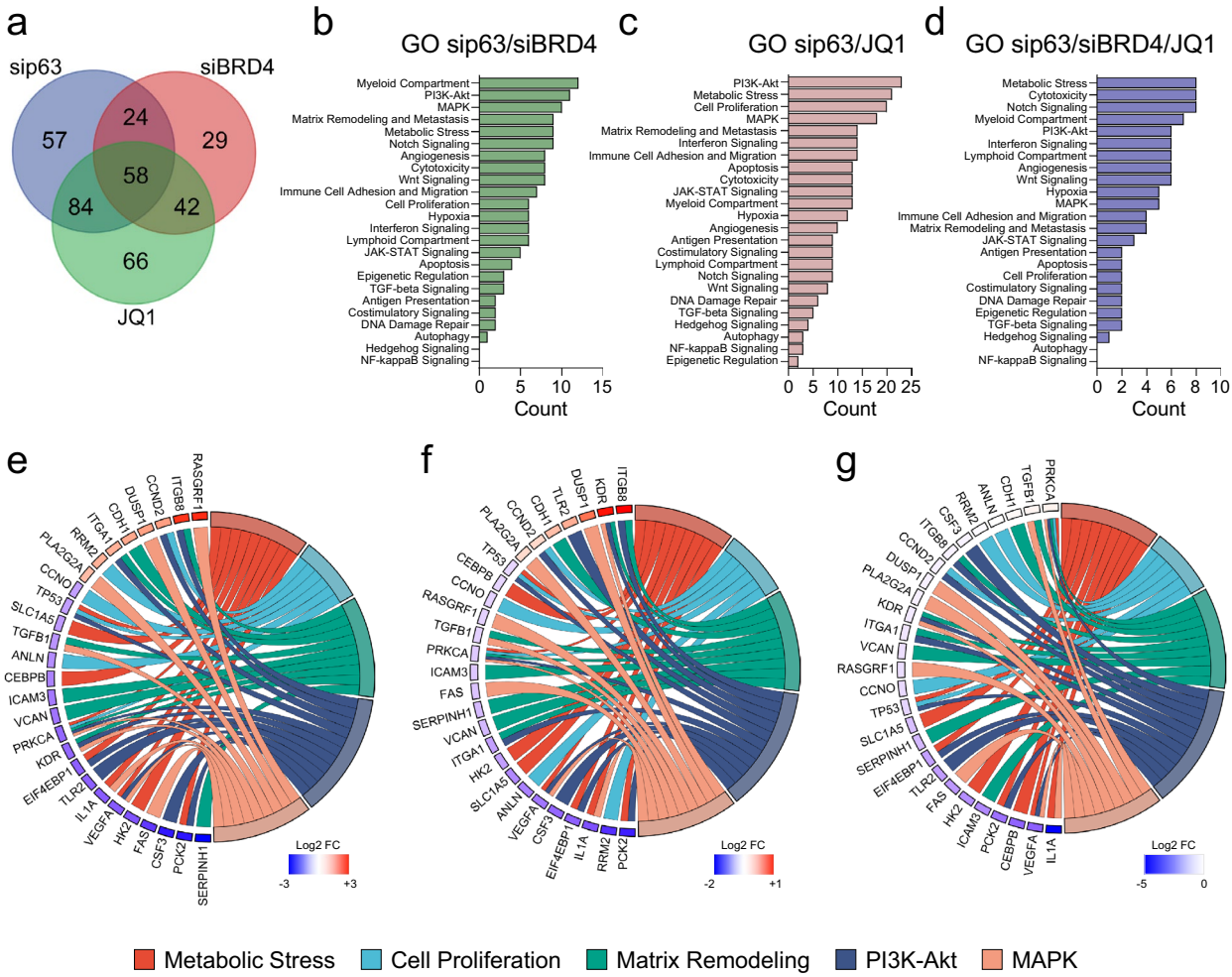

Fig. 4 Keratinocytes lacking for p63 and BRD4 have similar expression profiles. Gene expression analysis performed using nanoString technology on HEKn cells after p63 and BRD4 silencing and JQ1 treatment. **a** Venn diagram represents common dysregulated genes in the different conditions. **b**–**d** Pathway gene ontology of common dysregulated genes. **e**–**g** Expression levels of the shared dysregulated genes from the top five enriched categories of the GO-pathway analysis

Keratinocytes lacking for p63 and BRD4 have similar gene expression profile

To further investigate the transcriptional modulation mediated by BDR4 on p63 in keratinocytes biology, we decided to compare the changes in the transcriptomic profile after the silencing of p63 and BRD4 and BRD4 inhibition, using the nanoString technology. Specifically, we compared the expression of 770 selected genes belonging to different biological processes and tumor pathways (see materials and method section for further details).

We identified shared and unique differentially expressed genes (DEGs) across these three conditions, suggesting potential common pathways or mechanisms affected by these treatments. Gene ontology analysis revealed similarities in pathways modulation, such as cell proliferation, matrix remodeling and metabolic stress, abolishing the expression of p63 and/or BRD4 through specific RNAi (Fig. 4a) It's noteworthy to mention that similar results were obtained even inhibiting BRD4 activity with a specific inhibitor (Fig. 4c,d), accordingly to what we observed after p63 and BRD4 silencing(Fig. 4b). Furthermore, comparing the transcriptomic profiles of keratinocytes in absence of p63 (Fig. 4c) or BRD4 (Fig. 4d), or inhibiting BRD4 with JQ1 (Fig. 4e; Supplementary Fig. 3), we found that several dysregulated target genes were shared across the different conditions analyzed, showing a similar pattern of expression. Particularly, as expected, HK2 was downregulated in all the samples, confirming our previous results. Interestingly, we also found a downregulation of other crucial genes regulating keratinocytes homeostasis, as *TP53*, *VEGF* and *PCK2*, supporting our hypothesis that the co-binding of p63 and BRD4 is essential for supporting

p63-dependent transcription in keratinocytes, particularly for genes involved in metabolic stress, cell proliferation, matrix remodeling, and the PI3K-Akt/MAPK pathway(Fig. 4e–g).

Discussion

Our findings provide a comprehensive insight into the interconnected roles of p63 and BRD4 in keratinocyte biology. Our data suggest that p63-mediated transcriptional program is supported by the co-binding partner BRD4. The discovery that BRD4 interacts closely with p63 enhances our understanding of the chromatin landscape in keratinocytes during their proliferation and differentiation. Additionally, since BRD4 is well-known for its role in super-enhancer organization and transcription activator of different transcription factors, the results obtained provides relevant insights into the p63 interactome and its mechanism of action.

BRD4 recognizes and binds to acetylated histones, facilitating the transcriptional activation of various genes, including c-Myc [53], Devaiah BN et al. [54], which is essential for cell growth and proliferation. Therefore, in addition to the direct link between BRD4 and p63 in regulating the transcription of selected genes, BRD4 may also support keratinocyte proliferation and differentiation programs by engaging different transcriptional pathways, for instance the myc-dependent transcription program.

p63 and BRD4 pivotal roles in the maintenance and regulation of the skin's primary cell type, keratinocytes, suggest potential therapeutic implications, particularly in disorders where p63 is known to have pathological properties, such us psoriasis or epithelial tumors, including cutaneous and head and neck squamous cell carcinomas [55],Smirnov, Anemona, et al., 2019; [25, 26]. The use of JQ1, a specific BET inhibitor, to inactivate BRD4 activity [48], provides a hint towards the pharmacological interventions that could be employed. While this study elucidates the effects of JQ1 in keratinocyte proliferation and differentiation, its potential as an anti-cancer agent specifically targeting the p63-BRD4 axis remains to be fully explored in cutaneous disorders. Given that BRD4's inhibition mirrors the effects of p63 depletion in keratinocytes, BET inhibitors like JQ1 might be promising in conditions where p63 contributes to disease pathogenesis [56–58],Smirnov, Anemona, et al., 2019). Lastly, given that cutaneous squamous cell carcinoma originate mostly from keratinocytes, is worth to notice how in all the condition analyzed the Jak/Stat, PI3K-Akt and MAPK pathways are transcriptionally regulated by p63 and BRD4, suggesting that p63/BRD4 axis could also have a role in squamous cell carcinoma cancer formation and progression. Additionally, PI3K/Akt/mTOR pathway it's commonly dysregulated also in psoriasis [59, 60], an autoimmune disease resulting from an uncontrolled proliferation and aberrant differentiation of keratinocytes.

Considering these findings, future research endeavors should focus on delineating the precise molecular mechanisms through which BRD4 and p63 interact, especially in the context of skin diseases. Understanding this could provide a foundation for developing targeted therapies. Furthermore, the potential of BET inhibitors, alone or in combination with other agents, should be explored in preclinical and clinical settings, especially in tumors with aberrant p63 activity [56, 57]. In conclusion, the synergistic relationship between BRD4 and p63, two key players in keratinocyte biology, opens a new frontier in skin biology. This knowledge holds the promise of not only enhancing our understanding of skin pathologies but also of ushering in innovative therapeutic strategies.

Materials and methods

Cell culture, transfection, proliferation, and growth curve analyses

Primary Normal Human Epidermal Keratinocytes, neonatal (HEKn) (Gibco, catalog no. C-001-5C) and human TERT-immortalized keratinocytes (Ker-CT) (ATCC, CRL4048, lot. no. 0213) were cultured in EpiLife medium with the addition of Human Keratinocyte Growth Supplements (HKGS, Life Technologies). HEK293T (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. All cell lines were grown at 37 °C and 5% CO2 in a humidified atmosphere. Keratinocyte differentiation was induced by adding 1.2 mM CaCl2 to culture medium and the cells were collected at the following time points: 0, 3 and 6 days. For siRNA transfection, cells were seeded in 60 mm dishes plates at a density of 3×10^5cells per well and cultured overnight to reach 60–80% confluence. siRNAs targeting BRD4, p63 and negative control siRNAs were obtained from a commercial vendor (Merck, Sigma-Aldrich). Cells were transfected with 20 nM siRNA using Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The knockdown efficiency of BRD4 and p63 was evaluated by Western blotting or qPCR analysis 48 h after transfection. siRNAs are listed in Table S2. Plasmid DNA transfections were performed with Lipofectamine® 3000 (Invitrogen) according to the manufacturer's instructions. Plasmids pcDNA5-Flag-BRD4-WT and pCDNA5-Flag-BRD4-BD were a gift from Kornelia Polyak (Addgene plasmid #90,331 and #90,005)[51]. Plasmids p6346 MSCV-CMV-Flag-HA-Brd4-1–444 (Mut1), p6345 MSCV-CMV-Flag-HA-Brd4 1–722 (Mut2), p6347

MSCV-CMV-Flag-HA-Brd4-444–722 (Mut3) and p6348 MSCV-CMV-Flag-HA-Brd4 1047–1362 (mut4) were a gift from Peter Howley (Addgene plasmids #32,886, #31,352, 31,353 and #31,354 respectively) [52]. For JQ1 treatment, cells were seeded in 60 mm dishes plates at a density of 3×10^5cells per well and cultured overnight to reach 60–80% confluence. The culture media was than replace with fresh media supplemented with JQ1 (10 μM, Sigma, catalog no. SML1524) and the cells were collected 48 h after treatment. For cell cycle and proliferation analyses, cells were pulse-labeled with 10 μM EdU added directly to cell media, incubated for 2 h, and processed with a Click-iT EdU Alexa Fluor 488 flow cytometry assay kit (Invitrogen, catalog no. C10337). For both EdU assay and propidium iodide (PI), cells were stained and analyzed using a CytoFLEX flow cytometer (Beckman Coulter) using the appropriate filters and settings. Data were analyzed using CytExpert software (Beckman Coulter) and FlowJo software (BD Life Science). Cell growth was evaluated by seeding keratinocytes in 96 well-cell plates at a density of 3,000 cells/well after 48 h of transfection with p63 and BRD4 siRNAs. The Incucyte live-cell analysis system was used to measure cell growth in real-time capturing images every 3 h for 6 days. The Incucyte S3 software was used to analyze the confluence of the cells at different time points and normalized to the starting point of each sample. For the JQ1 treatment, cells confluence was monitored and analyzed after 48 h of treatment with 5μM and 10μM of JQ1. The medium was replaced every 48 h.

RNA extraction and RT-qPCR

Total RNA was extracted from cells using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Briefly, cells were harvested and lysed with Buffer RLT containing β-mercaptoethanol. RNA was then purified using a RNeasy Mini spin column and eluted in RNase-free water. The quantity and quality of RNA were assessed using a NanoDrop spectrophotometer. cDNA was synthesized from 1 μg of total RNA using the SensiFAST cDNA Synthesis Kit (Bioline) according to the manufacturer's instructions. qPCR was performed using QuantStudio™ 5 Real-Time PCR System and the Fast SYBR Green Master Mix (Applied Biosystems; catalogue A46109). The expression levels of target genes were normalized to the expression of the housekeeping gene human Tata Binding Protein (hTBP) using the $2^{-\Delta\Delta Ct}$ method. qPCR Primers used are listed in Table S1.

Chromatin immuno-precipitaion (ChIP) analyses

For Chromatin cross-linking immunoprecipitation (ChIP), N cells (5×10^6) were crosslinked for 10' in a solution containing 1% formaldehyde. After crosslinking, cells were lysed and sonicated to obtain chromatin fragments of ~ 300 bp. ChIP assays were performed using the Myers Lab ChIP-Seq protocol (Myers Lab, Hudson Alpha Institute of Biotechnology). The immunocomplex was immunoprecipitated using a specific anti-ΔNp63α (D2K8X, Rabbit mAb#13,109), anti-BRD4 (E1Y1P, Rabbit mAb #83,375) and non-specific IgG as negative control. Collected DNA fragments were tested both through semi-qPCR and Real Time-qPCR. Oligonucleotides are listed in Table S1.

Western blot

Cells were lysed in RIPA buffer containing Complete™ Protease Inhibitor Cocktail (Roche). The protein concentration was determined using the Protein Assay Dye Reagent Concentrate (Bio-Rad). Equal amounts of protein were separated on 10% SDS-PAGE gels and transferred onto PVDF membranes (Amersham Portran, GE Healthcare). The membranes were blocked with 5% non-fat milk in PBS-Tween for 1 h at room temperature and then incubated with primary antibodies against BRD4 (1:500; E2A7X, Rabbit mAb #13,440), p63α (1:1000, D2K8X, Rabbit mAb #13,109), K10 (1:1000; Biolegend, catalog no. 905404), Hexokinase II (1:500; Cell Signaling, C64G5 Rabbit mAb #2867), FOXM1 (1:500; sc-376620 Mouse, Envoplakin (1:500; sc-16747 Goat) and β-actin (1:50,000; Sigma A-5441) overnight at 4 °C. After washing with PBS-Tween, the membranes were incubated using the appropriate horseradish peroxidase-conjugated secondary antibody (rabbit and mouse; Bio-Rad, Hercules, CA, USA). Detection was performed with the ECL chemiluminescence kit (Perkin Elmer, Waltham, MA, USA) while the acquisition was performed with Alliance Q9 Advanced (Uvitec Cambridge). The protein expression levels were quantified using ImageJ software and normalized to those of β-actin. The uncropped images of the western blots are shown in supplementary figure S6.

Co-immunoprecipitation and western blotting

Co-immunoprecipitation was performed in the HEK293T cell line transfected for 24 h with corresponding plasmids. Whole-cell extracts obtained by lysing the cells with Triton buffer supplemented with Complete™ Protease Inhibitor Cocktail (Roche) were incubated were incubated with anti-FLAG M2 agarose beads ON at 4 °C (Sigma Aldrich). Endogenous immunoprecipitation was performed in HEKn cell line. Whole-cell extract was immunoprecipitated by incubating cells ON at 4°C with anti-ΔNp63α (D2K8X, Rabbit mAb #13,109) and

anti-BRD4 (E1Y1P, Rabbit mAb #83,375) primary antibody and then for 3 h at 4 °C with protein-G agarose beads (Roche). The beads were washed with Triton buffer, resuspended in loading buffer, and incubated at 98 °C for 10 min. For western blot assay, cells were collected and whole-cell protein extracts were obtained by lysing the cell pellet with RIPA buffer (50 mM Tris–cl pH 7.4; 150 mM NaCl; 1% NP40; 0.25% Na-deoxycholate;1 mM DTT), supplemented with Complete™ Protease Inhibitor Cocktail (Roche). The samples were loaded on an SDS–polyacrylamide gel and blotted on a PVDF membrane (Amersham Portran, GE Healthcare). Membranes were blocked with PBS-0.1% Tween 5% milk incubated with primary antibodies overnight at 4 °C, washed with PBS-0,1% Tween and hybridized for 1 h at RT using the appropriate horseradish peroxidase conjugated secondary antibody (MsxRb Light Chain specific HRP conjugated; GtxMs Light Chain specific HRP conjugated, Millipore). Immunoblotting was performed using the following primary antibodies: anti-Flag M2 (1:2000, F3165, Sigma), anti-HA (1:1000, Biolegend cat n°901,502) anti-p63α (D2K8X, Rabbit mAb #13,109) and anti-BRD4 (E2A7X, Rabbit mAb#13,440).

Proximity ligation assay (PLA)

Ker-CT cells were washed, fixed in formaldehyde and permeabilized with 0,1% Triton X-100 in PBS. The cells were probed with the following primary antibodies: p63 (1:500, Abcam #ab735), anti-BRD4 (E1Y1P) (Cell Signaling, Rabbit mAb #83,375). The PLA staining was performed with Duolink in Situ Red Starter Mouse/Rabbit kit (Sigma-Aldrich) according to the manufacturer's instructions. Images are obtained using Leica Stellaris 5 confocal microscope. ImageJ was used for the analysis.

Bioinformatic and statistical analysis

The gene expression analyses were performed using the panel "PanCancer IO 360" and run on the nCounter® Sprint Profiler. The data obtained were analyzed using the software nSolver 4.0 provided by nanoString technologies. All the analyses were performed following the manufacturer's instructions. The Gene and Pathway ontology analysis was performed with DAVID (https://david.ncifcrf.gov/home.jsp). The ChIP-seq analysis was performed with the online software "USCS Genome Browser" (https://genome.ucsc.edu/). TP63 binding profile was analyzed with the online database "Jaspar" (https://jaspar.genereg.net/). All statistical analyses were performed with Graph Pad Prism v8.0 software. The significance was calculated with Student's T-test. All results are expressed as means ± s.d. $P < 0.05$ was considered significant.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13062-024-00547-1.

Additional file 1.

Additional file 2.

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Author contributions

EC conceived and designed the research. EF and FR carried out bioinformatic analyses and prepared the first draft figures. EF, PR, AV and AML performed experiments in vitro. EF wrote the methods and results. GM and EC discussed the results, GM revised the draft. EC wrote the manuscript. All authors discussed the results.

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Declarations

Ethics approval and comsent to participate

Not applicable.

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