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## Research Article

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**Tetraspanin CD63 reduces the progression and metastasis of head and neck  
squamous cell carcinoma via KRT1-mediated cell cycle arrest**

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## **Abstract**

**Background:** Though metastasis is the leading cause of death in patients with head and neck squamous cell carcinoma (HNSCC), fundamental questions about the mechanisms that enable or inhibit metastasis remain unanswered. CD63, a tetraspanin family member, has been linked to tumor progression and metastasis. However, few studies have examined the role of CD63 in HNSCC.

**Methods:** We discovered CD63 expression in paired HNSCC tissue by tissue microarray. Cell phenotype experiments and lung metastasis mouse model were adopted to investigate the effect of CD63 on tumour metastasis in vitro and vivo. The molecular mechanism was investigated by mass spectrometry, Co-IP, immunofluorescence analysis and molecular docking.

**Results:** CD63 expression was abnormally decreased in HNSCC tissue compared to adjacent tissue, and that this was linked to prognosis. Overexpression of CD63 inhibited the progression and metastasis of HNSCC cells. KRT1 could be a direct interacting partner of CD63. CD63 reduces HNSCC progression via KRT1-mediated cell cycle arrest. Both CD63 and KRT1 expression was significantly decreased in metastatic tissue compared with primary tumor tissue.

**Conclusions:** We reveal a previously unrecognized role of CD63 in HNSCC cells. Our findings contribute to defining an important mechanism of HNSCC progression and metastasis.

**Keywords:** CD63; KRT1; metastasis; HNSCC; cell cycle arrest

## 1. Introduction

Head and neck squamous cell carcinoma (HNSCC) accounts for the vast majority of head and neck cancers [1]. The incidence of HNSCC has increased by approximately 30% since 1990, and it is now the seventh most common cancer worldwide [2]. Meanwhile, HNSCC patients are at a high risk of cervical lymph node metastases [3]. Cervical lymph node involvement is a well-known prognostic marker for HNSCC, and the presence of positive lymph nodes is thought to be a predictor of poor patient outcomes [4]. However, fundamental questions regarding the mechanisms that facilitate or restrain dissemination to local or distant metastases remain unrevealed.

Tetraspanins are transmembrane proteins, composed of outer loops and short N- and C-terminal tails with four transmembrane domains [5]. They form complexes known as tetraspanin-enriched microdomains by interacting with other tetraspanins and a variety of transmembrane and cytosolic proteins that are required for their function [6][7]. The tetraspanin protein CD63 has recently been described as a key factor in extracellular vesicles (EVs) production and endosomal cargo sorting [8][9]. Mounting evidence suggests that CD63 is also involved in tumor progression. The first work to link CD63 to cancer research can be traced back to melanoma research [10][11][12][13]. However, there is no consensus yet about whether CD63 promotes or blocks tumorigenesis. Lupia et al. established that CD63 is highly expressed in early melanoma and decreased in advanced lesions. CD63-silenced melanoma cells showed increased motor capacity and

aggressivity by regulating epithelial–mesenchymal transition (EMT)-related genes, suggesting that it is a possible suppressor of melanoma progression [6]. Kudo et al. showed the potential of cell surface CD63 to sensitize melanoma cells to BRAF inhibitor PLX4032 and to reduce the proliferation of PLX4032-resistant cells [14]. Yu et al. found that in hepatocellular carcinoma, overexpression of CD63 inhibited tumor cell proliferation and migration, whereas knockdown of CD63 promoted these phenotypes, possibly through CD63 regulation of IL-6/IL-27-STAT3 axis [15]. On the contrary, other studies have shown the opposite. Seubert et al. found that CD63 induced a more epithelial-like phenotype accompanied by increased E-cadherin expression and acted as a pro-metastatic factor [16]. However, few studies have shown the role of CD63 in HNSCC.

This study aimed to clarify the role of CD63 in the progression and metastasis of HNSCC in vitro and in vivo, to detect the correlation with clinical characteristics of patients with HNSCC, and to study the proteins that interact with CD63, exploring the mechanism of CD63-related phenotype changes in HNSCC.

## **2. Methods and materials**

### **2.1 Patient tissue and ethics approval**

Tissue microarray (TMA) was obtained from 80 patients (among the 160 cores, 22 were lost during processing) diagnosed with HNSCC pathologically after surgery from July 2014 to March 2018 (FDEENT TMA); paired fresh pathological specimens were obtained from 124 patients (including 13 pairs of primary and cervical lymph node

metastasis tissue) from March 2019 to June 2021 from the Department of Otorhinolaryngology, Eye & ENT Hospital of Fudan University. All participants provided written informed consent forms. This study was approved by the Ethics Committee of the Eye & ENT Hospital of Fudan University (NO.KJ2008–01). Commercial TMA (including 83 HNSCC tissue) was purchased from Superbiotek (ZL-HNT1021, Shanghai, China), which detailed information can be obtained from Supplementary Table 1.

## **2.2 Cell culture**

HuLa-PC, a cell line derived from posterior commissure of the larynx, was obtained from ATCC (Gaithersburg, Maryland) and cultured in Dermal Cell Basal Medium (ATCC® PCS-200-030™) supplied with Keratinocyte Growth Kit (ATCC® PCS-200-040™). The HNSCC cell line AMC-HN8 was a kind gift from Professor Sang Yoon Kim of Samsung Medical Center, Korea. Tu686 was obtained from Central South University (Hunan, China). FaDu and Detroit562 were obtained from the Cell Bank of the Shanghai Institute of Cells, Chinese Academy of Science (Shanghai, China). AMC-HN8 was cultured in RPMI-1640 (HyClone, Logan, UT). Tu686, FaDu, and Detroit562 were cultured in DMEM (Gibco, Grand Island, NY). The growth medium contained 1% penicillin-streptomycin (Genom Biotechnology, China) and 10% fetal bovine serum (FBS; Gibco, Grand Island, NY). The cells were incubated with 5% CO<sub>2</sub> at 37°C.

## **2.3 RNA isolation and qRT-PCR**

Total RNA was isolated from tissue and cells with TRIzol reagent (Invitrogen, Thermo Fisher Scientific) and then reversed-transcribed using an Evo M-MLV Mix Kit with gDNA Clean for qPCR (AG11728, Accurate Biology, Hunan, China). qRT-PCR was conducted using SYBR® Green Premix Pro Taq HS qPCR Kit (AG11718, Accurate Biology, Hunan, China) with the ABI 7500 Real-Time PCR System (Life Technologies, Shanghai, China). The primers were synthesized by Sangon Biotech (Shanghai). The sequences of primers used are as follows: CD63: ATGCAGGCAGATTTTAAGTGCT (forward primer), GTTCTTCGACATGGAAGGGATTT (reverse primer); KRT1: AGAGTGGACCAACTGAAGAGT (forward primer), ATTCTCTGCATTTGTCCGCTT (reverse primer); GAPDH: TGTAGTTGAGGTCAATGAAGGG (forward primer), ACATCGCTCAGACACCATG (reverse primer).

## 2.4 Immunoblotting analysis

Cell pellets obtained by centrifugation were incubated with RIPA lysis buffer (Beyotime, China) and a proteinase inhibitor cocktail (Yeasen Biotechnology Co., Ltd., Shanghai, China) for 30 min on ice. The lysates were then centrifuged at 14,000 rpm for 10 min, and the supernatants were collected and stored at -80°C. Lysate proteins were exposed to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane. After blocking with 5% (w/v) nonfat milk for 1 h, the membranes were incubated with primary antibodies overnight at 4°C. Primary antibodies used in immunoblotting analyses are listed in **Table 1**. The membranes were then

incubated with the relevant HRP-conjugated secondary antibodies. Specific antibody-bound protein bands were visualized using Azure Biosystems C280 (Azure Biosystems, Inc., USA). Gray value was analyzed by Image-pro plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

## **2.5 Immunofluorescence (IF) analysis and cell transfection**

IF analysis and cell transfection were performed according to our previous articles [17][18]. CD63 (ab59479, Abcam, 1:200 dilution) and cytokeratin 1 (sc-65999, Santa Cruz Biotechnology, 1:200 dilution) were used for the immunofluorescence analysis. Lentiviral transduction was used to induce gene expression or knockdown in HNSCC cells, with infection achieved by adding  $1 \times 10^7$  titer units of lentivirus to the culture media. CD63 overexpressing lentivirus and KRT1 knockdown lentivirus were purchased from Genomeditech (Shanghai, China). Lentivirus particles based on corresponding empty vectors were used as controls.

## **2.6 Sphere formation assay**

Suspension of  $1 \times 10^5$  cells were planted on ultra-low adhesive 24-well plates (EFL, Suzhou) and cultured in serum-free medium, containing DMEM-F12 (HyClone, Logan, UT), 2% B-27™ supplement (ThermoFisher Scientific, Waltham, MA), 20 ng/mL animal-free recombinant human EGF, 20 ng/mL human FGF-basic (154 amino acids), 10 ng/mL human LIF (all from PeproTech, Rocky Hill, NJ) and 1% penicillin-streptomycin

(Genom Biotechnology, China). Seven days after plantation, three fields of view in each well were randomly selected for imaging. The number of stem cell spheres was counted to assess sphere formation capacity.

## **2.7 Colony formation**

A suspension of  $1 \times 10^3$  cells was placed in each well of 6-well plates. When visible clones appeared in the well, the cells were fixed with 4% paraformaldehyde for 10 min, and then stained with 10% crystal violet for 10 min. The number of colonies was counted using Image-pro plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

## **2.8 EdU staining assays**

To analyze cellular proliferation, EdU staining was conducted using the BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 594 (Beyotime, Nantong, China). Briefly, the cells were incubated for 2 h at 37°C/5% CO<sub>2</sub> with 10 μM EdU. After incubation, the cells were washed twice with PBS and then fixed in 4% paraformaldehyde at room temperature for 10 min before being stained with DAPI for 5 min. After washing, the stained cells were captured using inverted microscope (Nikon inverted microscope ECLIPSE Ts2R, Japan). The ratio of EdU-positive cells represents the proliferation ability of the cells.

## **2.9 Migration assay**

Cells were maintained at 37°C with 5% CO<sub>2</sub> in 24-well plates, and migration was monitored at 10-min intervals for 24 h using an inverted OLYMPUS LCS-IX81 microscope. Images were captured using a 10x air objective with a pixel size of 0.625 µm. Data were combined from time-lapse image series collected from at least three independent experiments. Individual cells migrating were tracked, and their trajectories were obtained using the FastTrack AI automated analysis system Chemotaxis (MetaVi Labs/Ibidi, Germany).

## **2.10 Cell cycle assay**

Cells were starved for 24 h in serum-free culture medium to synchronize, then harvested during the logarithmic growth phase after another 12 h incubation. Single-cell suspensions were then fixed in 75% pre-cooling ethanol overnight at -20°C, washed twice with pre-cooling PBS, and then incubated with 500 µL PI/RNase staining buffer (BD Biosciences) for 15 min in dark. After filtering with a 40 µm mesh screen (BD Biosciences), cells were then tested using flow cytometry (SP8 MoFlo XDP, Beckman Coulter), and the cell cycle distribution was investigated with FlowJo Software (FlowJo LLC).

## **2.11 Immunoprecipitation (IP)**

Cells were lysed with 1000 µL ice-cold IP lysis buffer (Beyotime, Nantong, China) on ice for 30 min with periodic mixing. We then transferred the lysate to a microcentrifuge tube, which was centrifuged at 12,000 rpm for 15 min to pellet the cell debris at 4°C.

Protein A/G Magnetic Beads and Magnet Starter Pack (Thermo Scientific) were used to immunoprecipitate the magnetic beads-Ab-Ag complex, following the manufacturer's protocol. Mouse IgG (sc-2025, Santa Cruz Biotechnology) was used as a control. The proteins were separated by electrophoresis, and the binding protein was detected by immunoblotting analysis or liquid chromatography-tandem mass spectrometry (LC-MS/MS).

## **2.12 Mass spectrometry and co-immunoprecipitation assays (Co-IP)**

LC-MS/MS was performed using an EASY-nLC™ ab00 UHPLC system (Thermo Fisher) coupled with a Q Exactive HF mass spectrometer (Thermo Fisher) with the assistance of Shanghai Lu Ming Biotech Co., Ltd. (Shanghai, China). The Co-IP assay was carried out using a co-immunoprecipitation Kit (Millipore, Billerica, MD, USA) following the manufacturer's protocols. In brief, the cells were harvested and lysed and then incubated with IgG or specific for CD63 (sc-5275, Santa Cruz Biotechnology)/KRT1 antibody (sc-65999, Santa Cruz Biotechnology) rotated at 4°C overnight. Bead-bound proteins were released and analyzed by immunoblotting analysis.

## **2.13 Molecular docking**

Protein structure was obtained by Swiss-Model (<https://swissmodel.expasy.org/>). All protein structures are processed in the molecular operating environment (MOE 2019.1) platform, including removal of water and ions, protonation, addition of missing atoms,

complementing missing groups, and minimizing protein energy. Using HDock software, set the protein to rigid, the docking contact site to the full surface, the conformation generated after docking is set to 100, and the scoring function is used to select the conformation with the most negative energy.

#### **2.14 Gene set enrichment analysis (GSEA)**

GSEA was performed to elucidate KRT1-related pathways and gene sets in HNSCC. The gene expression profiles of 497 HNSCC samples were downloaded from The Cancer Genome Atlas (TCGA) datasets. According to KRT1 expression, the first 50% and last 50% samples were divided into high- and low-KRT1 groups, respectively. The number of permutations was 1000, and the threshold for the adjusted p-value was set to 0.05. A gene set is considered to be associated with KRT1 if most of its members are positively or negatively correlated with KRT1.

#### **2.15 In vivo metastasis assay**

For the in vivo metastasis assay, we injected  $1 \times 10^6$  cell suspensions in 200  $\mu$ L PBS into the tail vein in each group of 6-week-old male nude mice with a 29 G syringe needle and observed the survival of mice daily to obtain a survival curve. Eighty days post-injection, all mice that had not yet died were euthanized, and the lungs were dissected for subsequent histological assessment.

## 2.16 Immunohistochemical analysis (IHC)

IHC analysis was performed as previously described [17], with a slight modification. The tumor tissue excised from nude mice and patient specimens were fixed, dehydrated, and embedded in paraffin. Paraffin blocks were then sliced into 4- $\mu$ m sections and subjected to IHC, followed by 30 min of incubation with biotin-labeled secondary antibody. The positive number and staining intensity in each section were converted into corresponding H-score values to achieve semi-quantitative tissue staining.  $H\text{-Score} = \sum(p_i \times i) = (\text{percentage of weak intensity area} \times 1) + (\text{percentage of moderate intensity area} \times 2) + (\text{percentage of strong intensity area} \times 3)$ , where  $p_i$  represents the percentage of pixel area of positive signal;  $i$  for positive grade. The quantitative calculation process was completed by IHC Profiler plug-in in ImageJ software [19].

## 2.17 Microarray datasets

To verify the expression of CD63, microarray datasets GSE59102 (derived from the GPL6480 platform) and GSE83519 (derived from the GPL4133 platform) were selected from the GEO database (<http://www.ncbi.nlm.nih.gov/geo>). We downloaded the series matrix files and platform files. Each gene probe ID in the matrix files was converted to the gene symbol in the platform files by Perl. The expression of CD63 in each dataset was then extracted and calculated.

## 2.18 Statistical analysis

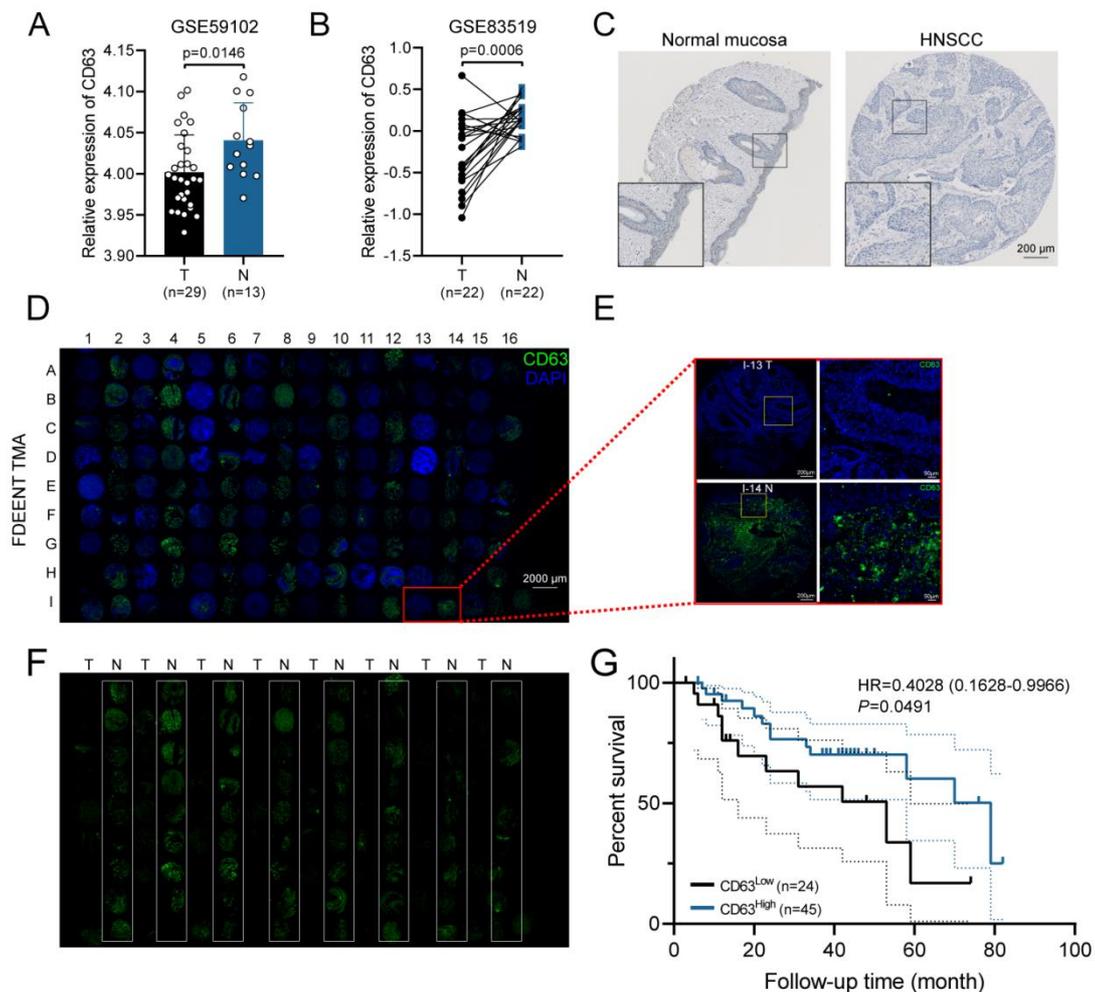
In each case, the data were pooled and averaged, and the standard deviation (SD) was calculated using GraphPad Prism (version 8; GraphPad Software, La Jolla, CA). Figure legends specify the *n* and error bars for each experiment. Student's t-test or one-way ANOVA was performed to evaluate the differences between two groups or more than two groups. Pearson's correlation was performed to analyze the correlation of gene expression. Differences were considered significant if the P value was <0.05 (\*), <0.01(\*\*), <0.001(\*\*\*), or <0.0001 (\*\*\*\*), as indicated in each figure legend.

## 3. Results

### 3.1 CD63 is downregulated in HNSCC tissue and is correlated with prognosis

To explore the expression of CD63 in HNSCC tissue, the GSE59102 and GSE83519 datasets were obtained from the GEO database and comprehensively analyzed, revealing that CD63 was significantly downregulated in HNSCC tumor tissue compared with normal tissue (**Figure 1A, 1B**). To verify this result, we performed TMA IHC staining, founding that CD63 is positive in the normal mucosa, but not in HNSCC tissue. (**Figure 1C, Supplementary Figure 1**). Further, Using 69 pairs of postoperative specimens from our cohort (FDEENT TMA), we validated that CD63 expression was downregulated in HNSCC tissue compared with adjacent tissue (**Figure 1D-F**), consistent with the GEO database and commercial TMA. We conducted a Kaplan–Meier survival analysis of 69

patients from our cohort. The results revealed that CD63 expression was positively associated with overall survival (OS) (**Figure 1G**). We then analyzed the correlation between CD63 and the clinical data. CD63 expression was significantly associated with lymph node metastasis ( $p = 0.041$ ) (**Table 2**). However, no correlation was observed between CD63 and clinical stage, tumor location, pathological differentiation, or recurrence. CD63 expression was also downregulated in two established HNSCC cell lines (AMC-HN8 and Tu686) compared with HuLa-PC, a human epithelial cell from the posterior commissure of the larynx (**Figure 2A, 2B**). These results showed that CD63 was significantly reduced in HNSCC tissue and is associated with prognosis and metastasis.



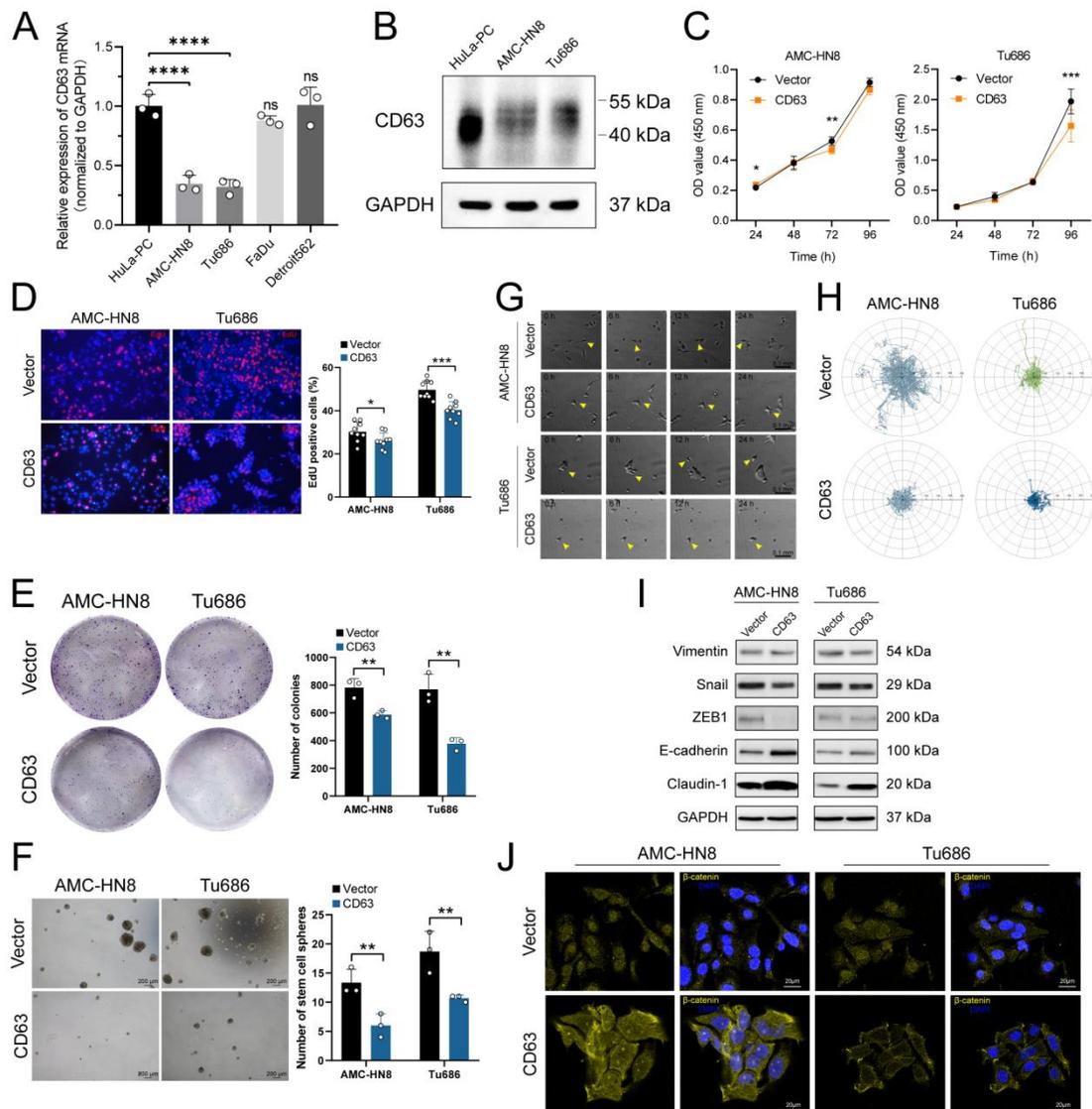
**Figure 1. CD63 is downregulated in HNSCC tissue and is correlated with prognosis.**

**(A)** CD63 mRNA expression levels were analyzed in HNSCC (T, Tumor, n = 29) and normal epithelial tissue (N, Non-tumor, n = 13) in the GSE59102 database. **(B)** CD63 mRNA expression levels were analyzed in paired HNSCC and adjacent tissue (n = 22) in the GSE83519 database. **(C)** Representative IHC staining image of CD63 in commercial TMA. Magnified images from the regions marked by rectangles were showed in the bottom. Scale bar, 200 $\mu$ m. **(D)** General observation of CD63 immunofluorescence staining in TMA among 69 HNSCC patients from our center. Scale bar, 2000  $\mu$ m. **(E)** Partially amplified images are shown in the frame. Scale bar, 50  $\mu$ m; CD63, green; DAPI, blue. **(F)** General observation of the difference between tumor and adjacent tissue. White rectangles, adjacent tissue. **(G)** Kaplan–Meier analysis of overall survival in patients with different CD63 expression subgroups.

**3.2 CD63 inhibits the proliferation, migration, stemness, and EMT of HNSCC cells in vitro**

We then characterized the phenotype landscape to fully demonstrate the function of CD63 in HNSCC in vitro. We designed and constructed lentiviral vectors that overexpressed CD63 in established HNSCC cell lines, AMC-HN8 and Tu686 cells. CCK8 proliferation assay, EdU staining assay, sphere formation assay, cell migration assays, and colony formation assays were performed to measure the effect of CD63 on HNSCC cell proliferation and migration. We found that overexpression of CD63 significantly

impaired the proliferation and migration properties of HNSCC cells (**Figure 2C–H**). We assessed the effect of CD63 on EMT via western blotting, which demonstrated that overexpression of CD63 significantly decreased the expression of Vimentin, Snail, and ZEB1, but dramatically enhanced the expression of E-cadherin and Claudin-1 (**Figure 2I**). This conclusion was further confirmed by confocal laser microscopy observation that the overexpression of CD63 disrupted the accumulation of  $\beta$ -catenin translocation from cytoplasm to nucleus, which is a key effector of EMT (**Figure 2J**).



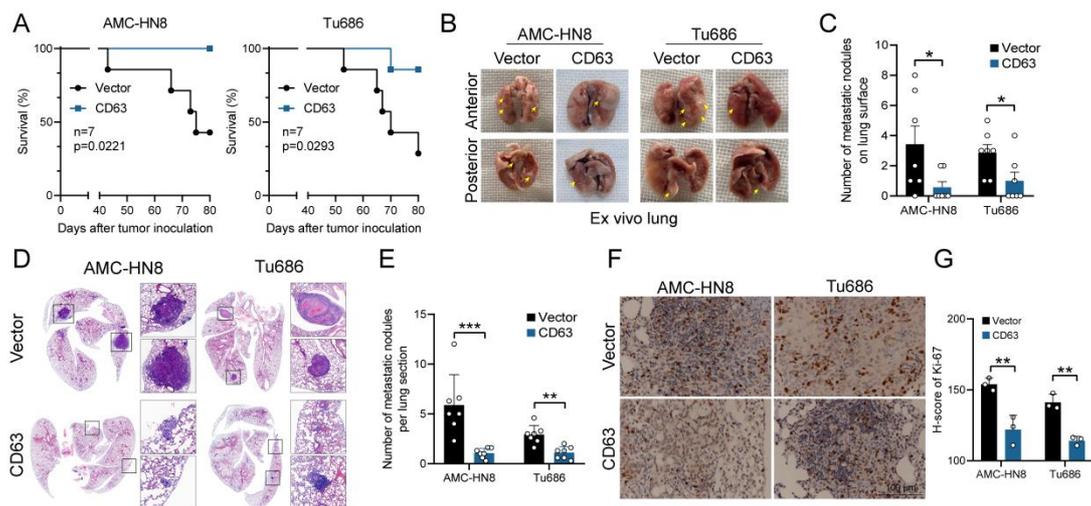
**Figure 2. CD63 inhibits the proliferation, migration, stemness, and EMT of HNSCC**

**cells in vitro. (A–B)** CD63 expression in HNSCC cell lines (AMC-HN8, Tu686, FaDu, and Detroit562) compared with that in the normal epithelial cell line HuLa-PC detected by qRT-PCR and western blotting. Cell proliferation assays **(C)**, EdU assays **(D)**, colony formation assays **(E)**, and sphere formation capacity **(F)** in the vector group and CD63-overexpressed AMC-HN8 and Tu686 cell groups. The right panel is the result of the quantitative analysis. **(G)** Representative images of each particular point in time in living cell imaging. The time span was 24 h. Scale bar, 0.1 mm. Yellow arrows, migrating cells. **(H)** Data were combined from time-lapse image series collected from at least three independent experiments. Individual cells migrating were tracked, and their trajectories were obtained using the FastTrack AI automated analysis system. **(I)** Western blot validation of EMT-related protein expression in the vector group and CD63-overexpressed group. **(J)** Representative images of the immunofluorescence analysis of  $\beta$ -catenin. Scale bar, 20  $\mu$ m. All data are shown as mean  $\pm$  SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ , \*\*\*\*,  $P < 0.0001$ .

### **3.3 CD63 reduces lung metastasis in vivo**

To investigate the role of CD63 in tumor metastasis in vivo, a lung metastasis model ( $n = 7/\text{group}$ ) was established. We injected different subgroups of HNSCC cells into nude mice via the tail vein and observed the survival of mice daily to obtain a survival curve. After 80 days, we found that the tumor-bearing mice in the CD63 overexpression subgroup had a better overall survival rate ( $p = 0.0221$  for AMC-HN8 cells,  $p = 0.0293$  for

Tu686 cells) (**Figure 3A**). The number and incident rate of metastatic nodules on the lung surface drastically decreased with CD63 overexpressed cells compared to the control cells (**Figure 3B, 3C**). The presence of pulmonary metastatic nodules of dissected lung tissue was also tested using H&E staining, with fewer metastatic nodules in the CD63 overexpression subgroup (**Figure 3F**). We also found that Ki-67 expression was lower in pulmonary metastases nodules tissue of the CD63 overexpressed subgroup (**Figure 3G**). These data provide evidence that CD63 is involved in reducing HNSCC cell metastasis in vivo.



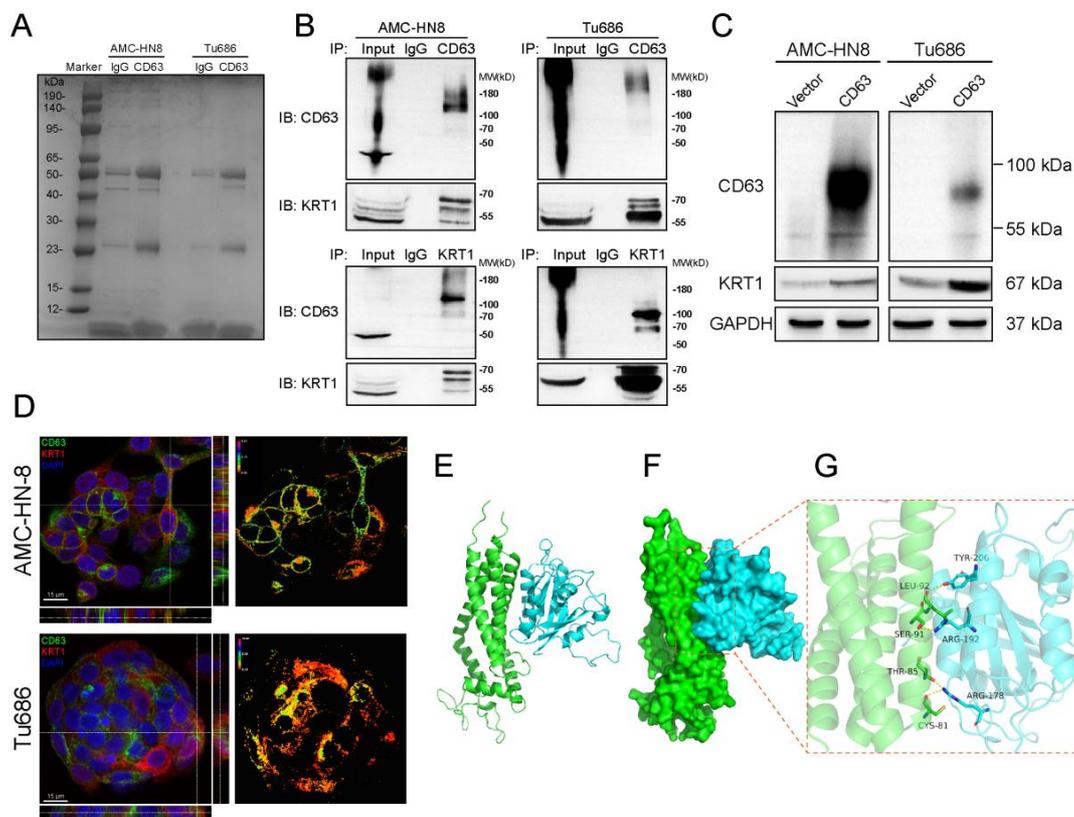
**Figure 3. CD63 reduces lung metastasis in vivo.** (A) Kaplan–Meier analysis of overall survival in tumor-bearing mice with different CD63 expression subgroups. Mice were transplanted with vector or CD63-overexpressed cells by tail vein injection (n = 7/group). (B–C) Representative images from each group were taken to observe and calculate the number of metastatic nodules on the lung surface. (D) H&E staining of lung sections. (E) The number of pulmonary metastases was counted or measured in random microscopic fields of each lung section. (F) Immunohistochemical staining was used to detect Ki-67

expression in pulmonary metastases tissue (n = 3). Scale bar, 100  $\mu$ m. **(G)** Quantitative analysis of H-scores of Ki-67. All data are shown as mean  $\pm$  SD. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001.

### 3.4 KRT1 is a direct interacting partner of CD63

To investigate the molecular mechanism underlying CD63-mediated suppression of HNSCC progression, we identified CD63-interacting proteins using mass spectrometry-based methods and co-localization image analysis. CD63 binding candidates were recognized by bands that were specifically found in CD63-overexpressing cells (**Figure 4A**). We found that KRT1, also known as keratin 1, was one of the prominent proteins in the eluent via mass spectrometry. To confirm the interaction of CD63 with KRT1, co-immunoprecipitation was performed. As expected, a positive KRT1 signal was observed in the immunoprecipitate pulled down using the anti-CD63 antibody. Reciprocal co-immunoprecipitation by anti-KRT1 antibody also resulted in the detection of CD63 (**Figure 4B**). The overexpression of CD63 in the cells was accompanied by increased KRT1 expression (**Figure 4C**). Furthermore, confocal microscopy showed that CD63 and KRT1 were co-localized in the cytomembrane of HNSCC cells (partly present in the cytoplasm in Tu686 cells) (**Figure 4D**). Molecular docking predicted that the binding score of CD63 and the KRT1 protein was -301.96 kcal/mol (**Table 3**). The binding sites of CD63 protein include LEU-92, SER-91, THR-85, CYS-81, and other amino acid residues. The binding sites of KRT1 include TYR-206,

ARG-192, ARG-178, and other amino acid residues. Thus, CD63 and KRT1 contact residues can form a variety of interactions, such as salt bridges, hydrogen bonds, hydrophobic interactions, and other interactions. These interaction forces can effectively improve the stability of the CD63 and KRT1 protein complexes (**Figure 4E–G**). These results imply direct physical interaction and co-localization of CD63 and KRT1 in HNSCCC cells.

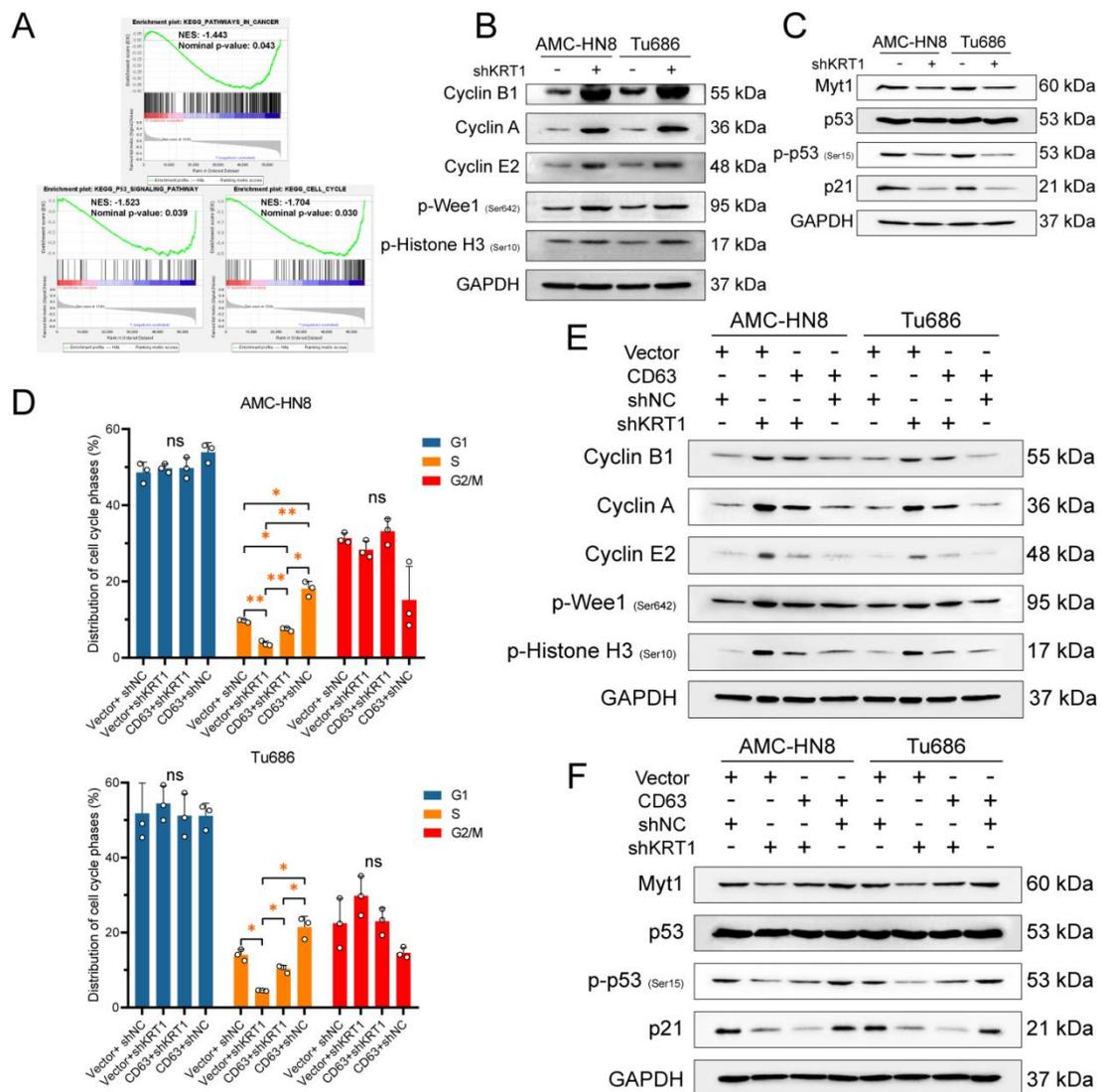


**Figure 4. KRT1 is a direct interacting partner of CD63. (A)** Coomassie blue staining of the immunoprecipitation pellet. **(B)** Co-IP assays of the CD63–KRT1 interaction. Cell lysates were precipitated (IP) with anti-CD63 antibody, and the precipitates were blotted (IB) with anti-KRT1 antibody (top). Conversely, cell lysate was precipitated with anti-KRT1 antibody and blotted with anti-CD63 antibody (bottom). Non-IP cell lysates (10%) were

used as input controls. **(C)** Western blot analysis of CD63 and KRT1 in the vector and CD63-overexpressed cells. **(D)** Representative orthogonal view of the immunofluorescence analysis of CD63 (green) and KRT1 (red) in the cells. Fluorescence ratio image (right panel) indicated colocalization and located in the region where CD63 and KRT1 interact. **(E–G)** Molecular modeling of a putative interaction between CD63 and KRT1. **(E)** The backbone of the protein was rendered in tube form and colored. **(F)** The electrostatic surfaces of CD63 (left) and KRT1 (right) proteins. **(G)** Detailed binding mode of CD63 with KRT1. The yellow dash represents a hydrogen bond or salt bridge.

### **3.5 CD63 reduces HNSCC progression via KRT1-mediated cell cycle arrest**

We found a significant relationship between cell cycle-related genes, especially p53 and KRT1, in HNSCC using GSEA (**Figure 5A**). Therefore, we used vitro models to explore the effects of silencing KRT1 on HSNCC cell cycle regulation. We found that the depletion of KRT1 altered cell cycle-associated genes and reduced the proportion of cells in the S phase of the cell cycle (**Figure 5B–D**), indicating that KRT1 functionally arrests the cell cycle of HNSCC. Subsequently, we found that overexpression of CD63 increased the proportion of cells in the S phase of the cell cycle, thereby blocking cell cycle progression, while simultaneous KRT1 depletion in cells overexpressing CD63 mitigated this effect (**Figure 5E, 5F**). These data prove that CD63 reduces HNSCC progression via KRT1-mediated cell cycle arrest.

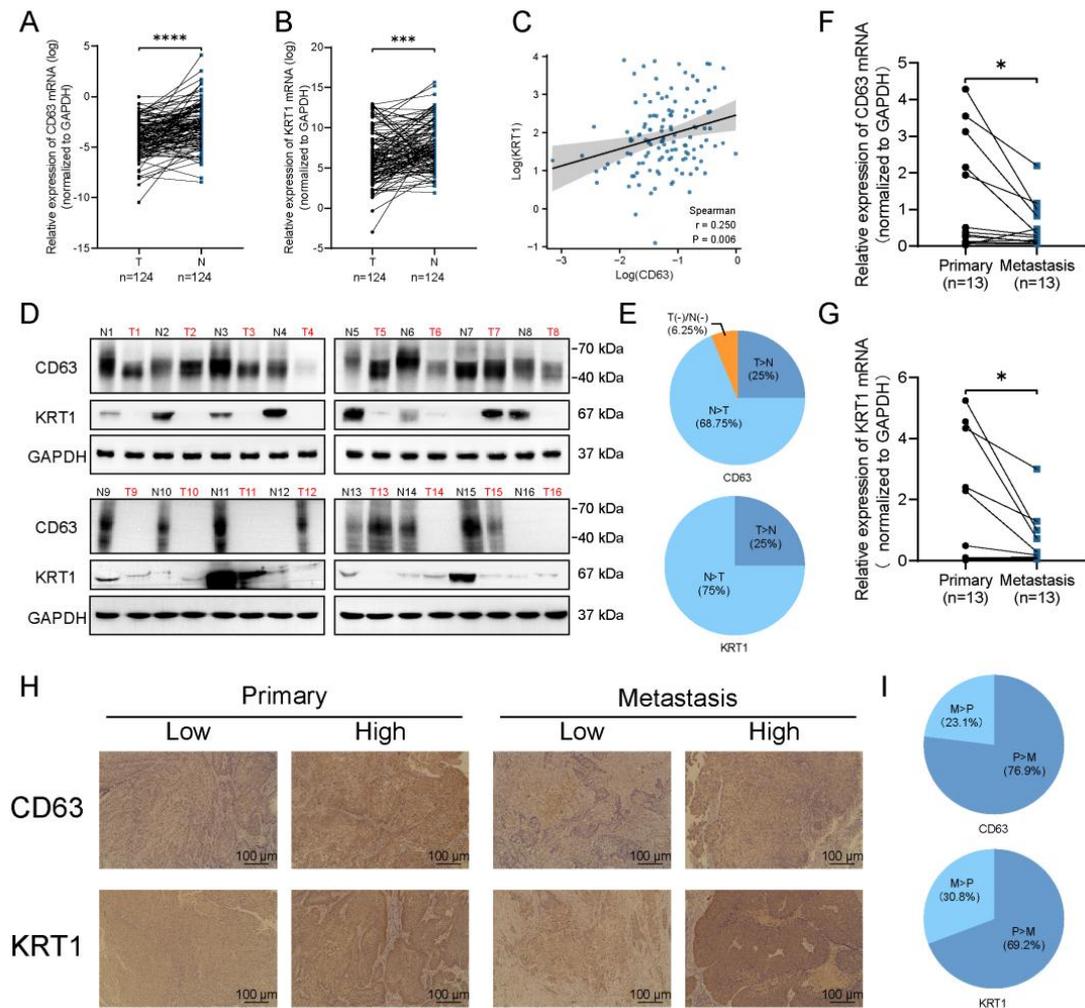


**Figure 5. KRT1 arrests the HNSCC cell cycle by mediating p53 in vitro.** (A) The GSEA results were plotted to visualize the correlation between the expression of KRT1 and dysregulated genes associated with the cell cycle and the p53 signaling pathway in HNSCC. (B–C) The protein levels of cell cycle regulation-related genes were detected by western blotting after KRT1 silencing. (D) Cell cycle assay was performed to show the properties of cell cycle regulation in the indicated subgroups. (E–F) The protein levels of cell cycle regulation-related genes were detected by western blotting in the indicated subgroups. All data are shown as mean  $\pm$  SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

### 3.6 The clinical relevance of CD63 and KRT1 in HNSCC

To examine the clinical impact of CD63 and KRT1 on HNSCC, we analyzed CD63 and KRT1 expression in paired tumors and adjacent normal (n = 124) tissue samples isolated from HNSCC patients. We found that the expression of CD63 and KRT1 in normal adjacent tissue was significantly higher than in paired tumor tissue (**Figure 6A, 6B**). The correlation analysis indicated a positive correlation between CD63 and KRT1 mRNA expression, suggesting that both proteins had the same inhibitory effect on HNSCC (**Figure 6C**). Furthermore, we verified these results by immunoblotting analysis of fresh paired pathological tissue (n = 16) (**Figure 6D, 6E**).

Moreover, to detect the expression of CD63 and KRT1 in primary and metastatic HNSCC tissue, we collected and tested tissue derived from the primary tumor region and cervical lymph node metastasis region (n = 13). As expected, we found that CD63 and KRT1 expression were significantly decreased in the metastatic region. These results suggest that CD63 and KRT1 reduce the metastasis of HNSCC (**Figure 6F-I**).



**Figure 6. The clinical relevance of CD63 and KRT1 in HNSCC. (A–B)** Comparison of CD63 and KRT1 expression in paired HNSCC tumor tissue and adjacent normal tissue by qRT-PCR (n = 124). **(C)** The correlation analysis indicated a positive correlation between CD63 and KRT1 expression. **(D)** Western blot analysis of CD63 and KRT1 protein expression in paired HNSCC tumor tissue and adjacent normal tissue (n = 16). **(E)** Quantitative calculation and statistics of the gray value. **(F–G)** Comparison of CD63 and KRT1 expression in paired primary HNSCC tumor tissue and cervical lymph node metastasis tissue by qRT-PCR (n = 13). **(H)** Representative images of immunohistochemical analysis of CD63 and KRT1 in paired primary HNSCC tumor tissue

(P) and cervical lymph node metastasis tissue (M). Scale bar, 100  $\mu$ m. (I) Quantitative calculation and statistics of the H-score. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ , \*\*\*\*,  $P < 0.0001$ .

#### 4. Discussion

In this study, we indicated that CD63 reduced the metastatic potential of HNSCC cells by regulating KRT1-mediated cell cycle arrest. Our findings show that the expression of CD63 had an impact on the intrinsic metastatic potential of HNSCC cells, as overexpression of CD63 produced decreased metastases. These observations support the notion of an anti-metastatic role of CD63 [20], which is also in line with clinical studies showing that CD63 is downregulated in tumor cells of cancer patients [21][22].

Our findings are in contrast to earlier reports suggesting a tumor-stimulative role of CD63, which studied the effect of CD63 in breast cancer cells [23] and other tumor cells [16]. Previous studies have indicated that CD63 might differ in its role during other stages of cancer progression, or it may play completely different or even opposite roles in various tumors [13][24][25]. CD63 is reportedly a highly N-glycosylated type III lysosomal membrane protein [26][27], and its glycosylation status may have a great influence on its role in tumor cells [23].

Although the predicted molecular weight of CD63 is 25 kDa [28], we found that the molecular weight of endogenous CD63 was 40–55 kDa (Figure 2B), while the molecular weight of CD63 of two HNSCC cells after CD63 overexpression shifted to 55–100 kDa (Figure 4C). Although the alteration between predicted and actual molecular weight can

be explained by glycosylation modification [6], the mechanism underlying the difference in molecular weight before and after the overexpression of CD63 still needs more research.

It is known that tetraspanin CD63 regulates cellular invasiveness through its association with peptidases and interacting proteins [5][7]. Jung et al. identified CD63 as a cell-surface binding partner of TIMP-1, regulating TIMP-1-driven cell survival and polarization by modulating the tetraspanin/integrin signaling complex [29]. In the present study, we identified KRT1 as the direct interacting partner of CD63 in HNSCC cells through co-localization studies, an hitherto unknown interaction. Similar to our finding, Suárez observed that CD63 polypeptide treatment changed the expression of KRT1, but no in-depth mechanistic studies were further conducted to explain this change [30]. Keratins, especially KRT1, the intermediate filament-forming proteins of epithelial cells [31], are extensively used as diagnostic biomarkers in cancers and are associated with tumorigenesis and metastasis in multiple cancers [32][33]. We found that KRT1, together with CD63, is prominently decreased in metastatic tissue compared with primary tumor tissue, which is consistent with findings in melanoma [34].

We believe that this is the first work to disclose a previously unrecognized role of CD63 in regulating KRT1-mediated cell cycle arrest in HNSCC cells, and our findings contribute to defining an important mechanism of HNSCC progression and metastasis.

## **Declarations**

## **Ethics approval and consent to participate**

The current study was approved by the Ethics Committee of the Eye & ENT Hospital of Fudan University (NO.KJ2008–01) and performed in strict accordance with the Declaration of Helsinki. All participants in this study signed informed consent documentation before sample collection. Animal experiments were approved by the Ethics Committee of the Eye & ENT Hospital of Fudan University and strictly performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Due efforts were made to limit animals' pain.

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

### **Competing interests**

The authors declare that they do not have any competing interests.

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### **Authors' contributions**

Conceptualization: QH, HLG, MZ and LZ; methodology: QH, YJS, CYH and YFZ; validation and formal analysis: QH, YJS, CYH and YFZ; investigation: QH, YJS, CYH, YFZ, YG and XHY; data curation: QH, YJS, CYH, YFZ and JYL; writing—original draft preparation: QH, YJS, CYH and YFZ; writing—review and editing: QH, YJS, CYH, YFZ, HLG, MZ and LZ; visualization: QH, YJS, CYH and YFZ; supervision: LT, HLG, MZ and LZ; funding acquisition: LT, HLG, MZ and LZ. All authors read and approved the final manuscript.

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### **Abbreviations**

HNSCC, head and neck squamous cell carcinoma; EVs, extracellular vesicles; EMT, epithelial–mesenchymal transition; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; IF, immunofluorescence; IP, immunoprecipitation; Co-IP, co-immunoprecipitation assays; GSEA, gene set enrichment analysis; TCGA, The Cancer Genome Atlas; IHC, immunohistochemical; TMA, tissue microarray; OS, overall survival

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