

Alleviation of Oxidative Stress Upregulates Protective Autophagy to Mediate PTX3-Induced Anoikis Resistance and Metastasis in HNSCC

Ben-Kuen Chen

bkchen58@mail.ncku.edu.tw

National Cheng Kung University <https://orcid.org/0000-0002-4720-4364>

Ting-Wei Chang

<https://orcid.org/0000-0001-9132-5828>

Wan-Chen Huang

Shih-Hung Chan

Jhih-Peng Tsai

Jing-Ting Chiou

Hsiao-Yu Chang

Ying-Zhu Meow

Tsan-Yu Chen

Ju-Ming Wang

National Cheng Kung University

Bing-Han Chung

Wen-Chang Chang

Taipei Medical University <https://orcid.org/0000-0002-1801-553X>

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Tables 1 to 6 are available in the Supplementary Files section.

1 **Alleviation of Oxidative Stress Upregulates Protective Autophagy to**
2 **Mediate PTX3-Induced Anoikis Resistance and Metastasis in HNSCC**

3 Ting-Wei Chang^{1,2}, Wan-Chen Huang³, Shih-Hung Chan⁴, Jhih-Peng Tsai^{2,4}, Jing-Ting
4 Chiou⁵, Hsiao-Yu Chang², Ying-Zhu Meow², Tsan-Yu Chen², Ju-Ming Wang⁶, Bing-
5 Han Chung⁷, Wen-Chang Chang^{5,#} and Ben-Kuen Chen^{1,2,#}

6

7 ¹ Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung
8 University, Tainan 701, Taiwan, ROC

9 ²Department of Pharmacology, College of Medicine, National Cheng Kung University,
10 Tainan 701, Taiwan, ROC

11 ³ Single-Molecule Biology Core Lab, Institute of Cellular and Organismic Biology,
12 Academia Sinica, Taipei 115, Taiwan, ROC

13 ⁴ Division of Cardiology, Department of Internal Medicine, National Cheng Kung
14 University Hospital, College of Medicine, National Cheng Kung University, Tainan
15 701, Taiwan, ROC

16 ⁵ Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical
17 University, Taipei 110, Taiwan, ROC

18 ⁶Department of Biotechnology and Bioindustry Sciences, College of Bioscience and
19 Biotechnology, National Cheng Kung University, Tainan 701, Taiwan, ROC

20 ⁷ Department of Otorhinolaryngology–Head and Neck Surgery, China Medical
21 University Hospital, Taichung 404, Taiwan, ROC

1 # **Correspondence to:** Ben-Kuen Chen, **email:** bkchen58@mail.ncku.edu.tw;
2 Tel. 886-6-2353535, Ext. 5470; Fax: 886-6-2749296;
3 ORCID : <https://orcid.org/0000-0002-4720-4364>
4
5 Wen-Chang Chang, **email:** wcchang@tmu.edu.tw;
6 Tel. 886-2-27361661, Ext. 3431; Fax: 886-2-66383879;
7 ORCID : <https://orcid.org/0000-0002-1801-553X>

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Abstract

Anoikis resistance (AR) is essential for the survival of circulating tumor cells for distant metastasis. However, the molecular mechanisms underlying AR survival and the promotion of further metastasis in head and neck squamous cell carcinoma (HNSCC) remain unclear. Here, we revealed that glycosylated PTX3 was enriched in AR cells in response to oxidative stress. The depletion of PTX3 significantly inhibited spheroid formation and survival, invasiveness, extravasation, and the formation of metastatic lung nodules in AR cells of HNSCC. Mechanically, moderate ROS levels in AR cells induced protective autophagy through the activation of EGFR, followed by upregulation of PTX3 expression regulated by the expression of ATG5/12, resulting in increased survivin expression. In contrast, inhibition of autophagy attenuated PTX3 expression and reduced anoikis resistance and metastasis. These results reveal that targeting protective autophagy-regulated factors, such as PTX3, can effectively inhibit HNSCC AR cell survival and metastasis.

Keywords: PTX3, anoikis resistance, ROS, protective autophagy, metastasis

1 **Introduction**

2 Head and neck squamous cell carcinoma (HNSCC) is the sixth most prevalent
3 cancer worldwide, with approximately a million new cases every year [1]. Despite the
4 ongoing advances in multiple therapeutic strategies and the reduced cigarette and
5 alcohol consumption of the population, 5-year survival rates remain under 50% [2].
6 Tumor metastasis, especially the regional state of lymph nodes, is among the most
7 important parameters for determining prognosis in patients with HNSCC. Even
8 detecting metastatic cancer cells in only a few lymph nodes can reduce patient survival
9 by as much as 50% [3, 4]. Unfortunately, prolonged survival is still unachievable in
10 patients with advanced HNSCC with regional or distal metastasis.

11 Recently, the metastatic dissemination of HNSCC via the lymphatic system has
12 garnered significant attention [5-7]. Upon detachment from the primary site, circulating
13 tumor cells (CTCs) face severe cellular stresses that typically trigger anoikis [8].
14 Nevertheless, distant metastasis is driven by a rare subpopulation of CTCs capable of
15 evading this anoikis-associated apoptotic process [9, 10]. Consequently, studying
16 anoikis-resistant (AR) cells is of paramount importance. Mounting evidence indicates
17 that an anoikis resistance gene signature, comprising genes such as *IKZF3*, *BAK1*,
18 *MTDH*, *FNI*, *PRDX4*, *ERBB2*, and *LTF*, strongly correlates with poor clinical
19 outcomes in HNSCC [11, 12]. However, the underlying molecular mechanisms that
20 enable AR cells to withstand detachment-induced stress and survive remain unclear.

21 Reactive oxygen species (ROS), which are typical byproducts of cellular stress
22 responses, play crucial roles in various physiological and pathological processes [13-
23 15]. Currently, the correlation between ROS production levels and cellular activity
24 remains controversial. Some studies have indicated that excessive ROS may induce
25 DNA double-strand breaks and inhibit cell viability [16]. Conversely, properly

1 regulated ROS can modulate cellular functions by oxidizing cysteine residues in
2 proteins and mediating processes such as NRF2-associated antioxidant responses,
3 AMPK-driven autophagy activation, and EGFR activation [17-20]. Interestingly, tumor
4 cells undergoing anoikis exhibit multiple distinct signatures, including upregulation of
5 apoptotic pathways and EMT markers, as well as increased ROS production and
6 autophagy activation [13-15]. Furthermore, although recent studies have linked NOX-
7 4-mediated ROS generation to EGFR expression during anoikis resistance in gastric
8 cancer [21], the precise mechanisms governing the levels of ROS and their downstream
9 effectors during suspension remain incompletely understood.

10 Autophagy, in response to ROS modulation, maintains cell survival during
11 suspension. However, previous studies have indicated that autophagy activation is
12 intimately linked to EGFR activation [22, 23]. Our previous studies revealed that EGFR
13 activation promotes HNSCC anoikis resistance and metastasis via various molecules,
14 including PTX3 [24-27]. Furthermore, a correlation between PTX3 and both oxidative
15 stress and autophagy activation has been reported [28-30]. Notably, analysis of tissue
16 specimens from HNSCC patients revealed significantly higher PTX3 expression in
17 malignant and metastatic tissues than in normal tissues [26, 31]. However, the
18 correlation between PTX3 expression and anoikis resistance, which is regulated by
19 cellular anti-apoptotic pathways such as autophagy, remains unclear.

20 In this study, we demonstrate that suspension-induced cellular stress promotes
21 cytotoxic autophagy via excessive accumulation of ROS, ultimately leading to cell
22 death. However, in anoikis-resistant cells, they exhibited altered ROS levels,
23 accompanied by increased EGFR activity, leading to a cellular response toward
24 protective autophagy and upregulation of PTX3 expression with glycosylation.
25 Specifically, glycosylated PTX3 markedly enhances anoikis resistance by upregulating
26 survivin expression, whereas its continuous upregulation further promotes tumor cell

- 1 metastasis after reattachment. These findings provide new insights into therapeutic
- 2 strategies involving targeting PTX3 induced by protective autophagy to eliminate
- 3 circulating HNSCC cells.

1 **Materials and Methods**

2 **Data acquisition and bioinformatics analysis**

3 Data regarding mRNA expression correlations, clinicopathological association
4 analyses, and patient survival rates for Head and Neck Squamous Cell Carcinoma
5 (HNSC) were obtained from The Cancer Genome Atlas (TCGA-HNSC) dataset.
6 Proteomic data were retrieved from the Clinical Proteomic Tumor Analysis Consortium
7 (CPTAC) dataset. Data concerning PTX3 knockdown (shPTX3) across various cancer
8 cell lines were sourced from the Cancer Dependency Map (DepMap) project. The
9 validating geneset of HNSCC anoikis-resistant signatures was curated and extracted
10 from previously published literature. The data were processed and analyzed utilizing
11 the GEPIA2 (Gene Expression Profiling Interactive Analysis 2) web server and the Q-
12 OMICs platform. Gene expression datasets comparing different cancer cell types
13 cultured under suspension versus adherent (attached) conditions were acquired from
14 the Gene Expression Omnibus (GEO) database. Differential gene expression analysis
15 between these two distinct physical conditions was performed using the GEO2R
16 interactive web tool.

17 **Cell culture and reagents**

18 FaDu head and neck cancer cell line was purchased from Bioresource Collection
19 and Research Center (BCRC, Hsinchu, Taiwan). Human microvascular endothelial cell
20 line (HMEC-1) were purchased from the American Type Culture Collection (ATCC,
21 Manassas, VA, USA). The head and neck cancer cell lines TU183, HSC-3, OECM-1,
22 UM-SCC1, SAS, SCC-4, SCC-15, SCC-25, Detroit-562 and HONE-1 were kindly
23 provided by Dr. Kwang-Yu Chang (National Health Research Institutes, NHRI, Taiwan).
24 FaDu, HSC-3 and Detroit-562 cells were maintained in a ATCC-modified MEM culture
25 medium (Corning). SCC4, SCC-15 and SCC-25 were maintained in DMEM/F12
26 medium with 400 ng/ml hydrocortisone. UM-SCC1, SAS and TU183 cells were

maintained in a DMEM-high glucose culture medium (Gibco). HONE-1 and OECM-1 cells were maintained in ATCC-modified RPMI 1640 culture medium (Gibco). HMEC-1 cells were maintained in an MCDB131 culture medium (GeneDireX). All cell lines were maintained at 37°C in 10-cm plastic dishes containing 8 ml medium. Each medium was supplemented with 10% fetal bovine serum (Corning), 100 µg/ml Streptomycin (Sigma-Aldrich), and 100 units/ml Penicillin (Sigma-Aldrich).

N-acetylcysteine (NAC) was purchased from Sigma-Aldrich and dissolving in ddH₂O, followed by sterilization through a 0.22 µm filter.. Metformin was prepared by dissolving commercial tablets (Metose F.C.) in ddH₂O, followed by sterilization through a 0.22 µm filter. FCCP and rotenone were obtained from Abcam. The pharmacological compounds and inhibitors, including 3-methyladenine (3-MA), bafilomycin A1 (Baf-A1), chloroquine (CQ), parthenolide, SP600125, LY294002, U0126, Y15, tunicamycin, NGI-1, rapamycin, gefitinib, and erlotinib, were acquired from MedChemExpress (MCE) and dissolved in DMSO.

Tumor spheroid formation assay

For the cell selection phase, 10-cm culture dishes were coated with poly-HEMA to inhibit cell adhesion. Single-cell suspensions were seeded into these dishes and cultured in suspension for seven days. Following each incubation period, the culture medium containing the cells was collected, and cellular debris was removed via centrifugation. The isolated spheroids were subsequently transferred to standard culture dishes to allow for recovery and reattachment. Upon the resumption of normal growth, the cells were subjected to another round of suspension culture. This process was repeated a minimum of three times prior to evaluating the viability of the spheroids. For the generation of small-scale spheroids, 6-well or 96-well ultra-low attachment plates were utilized (Corning). For morphological evaluation, bright-field images of the tumor spheroids were acquired under a light microscope. The diameters of the spheroids

1 were then measured and quantified utilizing ImageJ software. The cell concentration
2 was kept consistent across the different plate formats.

3 **Mitochondrial superoxide, cell survival and autophagy status detection**

4 Cell spheroids were collected and centrifuged to remove dead cells and cellular
5 debris. The supernatant was discarded, and the spheroids were dissociated into single
6 cells using Accumax reagent. Following incubation, the reaction was neutralized with
7 FBS-containing culture medium, and a single-cell suspension was obtained via
8 centrifugation. The cells were then divided for subsequent staining. For mitochondrial
9 superoxide assessment, cells were resuspended in PBS containing 5 μ M MitoSOX
10 (Thermo). To detect autophagy or apoptosis, cells were resuspended in 1X assay buffer
11 containing either CYTO-ID (Enzo Life Science) or Annexin V/PI ((BD Biosciences)
12 staining reagents, respectively. After a 30-minute incubation in the dark in a cell culture
13 incubator, the samples were analyzed by flow cytometry. MitoSOX and CYTO-ID
14 fluorescence signals were acquired using the PE and FITC channels, respectively.
15 Annexin V and PI signals were also detected in the PE and FITC channels, with
16 appropriate compensation applied to correct for spectral overlap.

17 **RNA sequencing and bioinformatics analysis**

18 Total RNA purity and integrity were assessed using a SimpliNano
19 Spectrophotometer (Biochrom) and a Qsep 100 DNA/RNA Analyzer (BioOptic Inc.).
20 Sequencing libraries were constructed from 1 μ g of total RNA per sample using the
21 KAPA mRNA HyperPrep Kit (Roche) according to the manufacturer's protocol. The
22 libraries were amplified via PCR, and their final quality was validated using a Qubit
23 2.0 Fluorometer and an Agilent Bioanalyzer 2100.

24 The qualified libraries were sequenced on an Illumina NovaSeq X platform to
25 generate 150-bp paired-end reads. Raw data quality was evaluated using FastQC and
26 MultiQC. Trimmomatic was utilized to remove adapter sequences and low-quality

1 bases. The resulting high-quality clean reads were aligned to the human reference
2 genome (GRCh38) using HISAT2, and read counts per gene were quantified using
3 featureCounts.

4 Gene expression normalization and differentially expressed gene (DEG) screening
5 were performed using DESeq2. The resulting P-values were adjusted using the
6 Benjamini-Hochberg approach to control the false discovery rate (FDR). Functional
7 enrichment analyses, including Gene Ontology (GO) and KEGG pathways, were
8 conducted using the clusterProfiler package, while Gene Set Enrichment Analysis
9 (GSEA) was performed against the MSigDB with 1,000 permutations. Finally, the
10 relevant figures were generated using a multi-omics platform (Biotools).

11 **Reverse transcription quantitative polymerase chain reaction (RT-qPCR)**

12 The total RNA of cells was extracted from adherent cells/ spheroids by using
13 REzol C&T (PROtech). A reverse transcriptase reaction was performed using a high-
14 capacity cDNA transcription kit (Thermo) according to the manufacturer's instructions.
15 Following cDNA synthesis, gene-specific primers were designed using NCBI primer
16 design software. Reverse transcription quantitative PCR was performed in triplicate
17 using PowerUp™ SYBR™ Green Master Mix (Thermo) and the StepOne Plus RT-
18 qPCR System (Applied Biosystems). Each well contained the following reaction
19 mixture: 5 µl of 2 × PowerUp™ SYBR™ Green Master Mix, 4 µl of RNase-free water
20 containing 30 ng cDNA, 100 nM sense, and antisense primers. Universal cycling
21 conditions were used. Relative gene expression was calculated using the comparative
22 Ct method. All values were normalized to the housekeeping gene GAPDH. The specific
23 primer sequence was previously described.

24 **Western blotting**

25 An analytical 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis
26 (SDS-PAGE) was performed, and 30 µg of protein was analyzed. For immune blotting,

1 an electroblotting apparatus transferred proteins in the SDS gels onto polyvinylidene
2 difluoride membranes (Millipore). Antibodies against human phospho-EGFR^{Y1068},
3 ATG5, survivin, Beclin-1, SQSTM-1/p62 phospho-AKT^{T308} and LC3B (Cell Signaling
4 Technology); PTX3, total EGFR and GAPDH (Santa Cruz Biotechnology); vimentin
5 (BD Biosciences); MMP3, MMP9, ATG16L1, ATG12, ATG7, p84 phospho-ERK
6 T202/Y204 + ERK2-T185/Y187 and phosphor-BCL2^{S70}(ABclonal Teconology) and p-FAK^{Y397}
7 (Thermo- Fisher Scientific) were used as the primary antibodies. Mouse or rabbit IgG
8 antibodies coupled to horseradish peroxidase were used as secondary antibodies. An
9 enhanced chemiluminescence kit (Millipore) was used for detection using the iBright
10 Imaging System (Thermo).

11 **Immunofluorescence**

12 For adherent cells, the cells were seeded onto chamber slides and fixed with 4%
13 paraformaldehyde in phosphate-buffered saline (PBS) at 25°C for 30 min. Following
14 three washes with PBS, the cells were permeabilized and blocked using a solution
15 containing 1% Triton X-100 and 2% fetal bovine serum (FBS) for 15 min. Next, the
16 cells were incubated with the indicated primary polyclonal antibodies (1:100 dilution)
17 for 1 h. Subsequently, the cells were treated with Alexa Fluor® 546-conjugated goat
18 anti-rabbit IgG secondary antibodies (1:500 dilution), with or without Alexa Fluor®
19 488 Phalloidin (1:200 dilution; Invitrogen, Life Technologies, Carlsbad, CA, USA), for
20 30 min. Finally, the cells were counterstained with 0.2 µg/µL 4',6-diamidino-2-
21 phenylindole (DAPI; Invitrogen) and mounted using a 90% glycerol mounting solution.
22 Digital images were captured using an Olympus FV3000 confocal microscope
23 (Olympus America, Inc., Melville, NY, USA). For suspension cells, staining reagents
24 were added directly to the ultra-low attachment dishes containing the spheroids. The
25 samples were then incubated on a shaker in the dark for 30 min, and images were
26 acquired utilizing an Olympus APX100 microscope.

Matrix metalloprotease (MMP) activity assay

The 50 μ L of conditioned media harvested from HNSCC adherent cells/ spheroids were transferred to a black 96-well plate and followed by the procedure of Amplite Universal Fluorimetric MMP Activity Assay Kit (Green fluorescence, AAT bioquest). Briefly, 2 mM amino-phenyl mercuric acetate (APMA) was mixed with a conditioned medium and then incubated at 37°C for 3 h. After incubation, 100 μ L of green substrate was then added into each well and further incubated for 3/ 24 hours. The fluorescence intensities of the background and samples were measured using SpectraMax iD3 Multi-Mode Microplate Reader (Ex: 495 nm, Em: 525 nm).

Plasmid construction

The wildtype PTX3 was constructed into pcDNA3.1(+)_myc_His A vector (OmicsBio) using KpnI and XbaI restriction enzymes. The 220A mutant of PTX3 was constructed by the site-directed mutagenesis method as previously described. The vector sequence was confirmed by DNA sequencing.

Transfection of cells with siRNA oligonucleotide or plasmids

Transient transfection of cells with 40 nM siRNA oligonucleotides and plasmids was performed using RNAiMAX and Lipofectamine 2000 (Invitrogen), respectively, according to the manufacturer's instructions with slight modifications. The siRNA IDs were as follows, Scramble (siRNA ID: D-001810-10-50), BIRC5 (Survivin, siRNA IDs: L-003459-00-0005) (Dharmacon); PTX3 (siRNA IDs: S11586, S11587) (Invitrogen); ATG5 (siRNA IDs: SC-41445); ATG7 (siRNA IDs: SC-41448); ATG12 (siRNA IDs: SC-72578); ATG16 (siRNA IDs: SC-72580). For transfection, the lipid complex was diluted in Opti-MEM medium (Invitrogen) and incubated with cells for 24 hours. After incubation, the medium was replaced with fresh culture medium to conduct subsequent experiments.

Transwell migration and invasion assay

Transwell migration and invasion assay was performed using transwell inserts (polyethylene terephthalate membranes with 8 μ m pores, Millipore). Briefly, for the migration assay, cells (5×10^4) were seeded in upper chamber containing a serum-free medium and a lower chamber containing a 10% FBS culture medium. After 24 hours, cells in the upper chamber were removed, and migrative cells at the bottom of the membrane were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet (Sigma-Aldrich). Crystal violet staining cells were then solubilized with 10% acetic acid, and absorbance (OD, 595 nm) was measured using SpectraMax iD3 Multi-Mode Microplate Reader. For invasion assay, cells were seeded in the upper chamber coated with 1:10 dilution of Matrigel in a 2% FBS culture medium. After 48 hours, the invaded cells were measured as the description above.

Matrix metalloprotease (MMP) activity assay

The 50 μ L of conditioned media harvested from HNSCC adherent cells/ spheroids were transferred to a black 96-well plate and followed by the procedure of Amplitude Universal Fluorimetric MMP Activity Assay Kit (Green fluorescence, AAT bioquest). Briefly, 2 mM amino-phenyl mercuric acetate (APMA) was mixed with a conditioned medium and then incubated at 37°C for 3 h. After incubation, 100 μ L of green substrate was then added into each well and further incubated for 3/ 24 hours. The fluorescence intensities of the background and samples were measured using SpectraMax iD3 Multi-Mode Microplate Reader (Ex: 495 nm, Em: 525 nm).

Cell adhesion assay

HMEC-1 cells were seeded into the channel slides (Ibidi) coated with fibronectin (F4759, Sigma-Aldrich) for 24 h to form a monolayer. Tumor cells were treated with 50 ng/ml EGF for 3 h and then labeled with 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI) (Invitrogen). DiI-labeled tumor cells were added to the HMEC1 monolayer for 30~60 min and then gently washed with PBS

1 to remove non-adherent cells on slides and fixed with 4% PFA. The adherent cells were
2 quantified by flow cytometry. The experiments were conducted in a humidified 5% CO₂
3 incubator at 37°C.

4 **Tumor extravasation and metastasis assay in an animal model**

5 To evaluate tumor metastasis, an in vivo tail vein injection model was established
6 using 8-week-old male severe combined immunodeficient (SCID) mice. For both the
7 tumor extravasation and lung nodule formation assays, 5×10^5 DiI-labeled cells (1,1'-
8 dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) suspended in 100 µL of
9 PBS were intravenously injected. In the extravasation assay, mice were euthanized
10 within 48 hours post-injection. Lung tissues were fixed in 4% paraformaldehyde,
11 cryoprotected with 30% sucrose, and embedded in FSC 22 (3801480, Leica) for
12 cryosectioning at 8 µm. Immunohistochemical (IHC) staining with an anti-CD31
13 antibody (ab28364, Abcam) was performed to identify blood vessels. For the nodule
14 formation assay, mice were euthanized 8 weeks post-injection. Lung tissues were
15 paraffin-embedded, sectioned, and subjected to H&E staining. Whole-slide imaging
16 was conducted using a BX63 upright microscope. All animals were sourced from the
17 National Cheng Kung University (NCKU) Laboratory Animal Center (Tainan, Taiwan),
18 and all procedures were approved by the Institutional Animal Care and Use Committee
19 (IACUC) of NCKU Medical College (Approval No. NCKU-IACUC-113-207).

20 **Statistical analysis**

21 Statistical analysis was performed with either t-test (for comparison between two
22 groups) or a one-way ANOVA analysis of variance (Tukey's or Newman-Keuls' post-
23 tests, for comparison among multiple experimental groups) using GraphPad Prism 6.0
24 software (GraphPad Software, San Diego, CA, USA). The in vitro data are presented
25 as the mean $\pm$ standard deviation (SD) determined from three independent experiments.
26 A Fisher's exact test and Kendall's tau (τ)-b correlation analysis were used to examine

1 the relationship between the expression levels of PTX3 and various clinicopathologic
2 features. A P-value less than 0.05 shows the significance and is denoted by an asterisk
3 (*). The P-values less than 0.01 and 0.001 are indicated by ** and ***, respectively.
4 n.s.: no significant difference.

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Results

Clinical relevance of anoikis-related genes and prognosis in HNSCC

Anoikis is a form of programmed cell death induced when cells lose contact with the extracellular matrix, and it plays a critical role in regulating a range of physiological and pathological conditions. Notably, resistance to anoikis has increasingly been recognized as a hallmark of cancer, particularly in integrin-overexpressing melanoma and oral cancers [12, 32, 33]. To investigate the potential association between anoikis and clinical outcomes in cancer patients, we used the Q-OMICS platform to analyze the correlation between the expression of the anoikis-related gene (ARG) signature (GO:0043276) and overall survival in patients from the Cancer Genome Atlas (TCGA) database [34]. As shown in Table 1, our pancancer analysis revealed that high ARG expression was specifically associated with improved overall survival in patients with HNSCC and cutaneous melanoma (SKCM). Using the staging criteria for HNSCC provided by the American Academy of Otolaryngology–Head and Neck Surgery (AAO-HNS), patients were stratified into early-stage (Stage I–II) and advanced-stage (Stage III–IV) groups. Furthermore, both the TCGA and Clinical Proteomic Tumor Analysis Consortium (CPTAC) databases revealed that the transcription and translation levels of the anoikis signature were significantly enriched in advanced-stage HNSCC patients (Supplementary Fig. 1A–B). Additionally, stratification of patients based on different cutoff values for anoikis signature expression revealed that individuals with high ARG expression exhibited significantly better overall survival and disease-free survival, particularly in advanced-stage patients (Supplementary Fig. 1C–D).

Given that the anoikis signature comprises both positively and negatively regulated genes, we further investigated the expression of individual anoikis-related genes (ARGs) in patients with advanced-stage cancer to clarify their specific impact on clinical outcomes. Through a comprehensive literature review, we categorized these

1 genes on the basis of their roles in manipulating anoikis. Notably, high expression of
2 the BMF and TSC2 genes, which are associated with the promotion of anoikis, was
3 significantly correlated with improved prognosis in patients with stage III/IV disease
4 compared with those with stage I/II disease (Table 2 and 3). In contrast, the
5 overexpression of genes known to confer anoikis resistance, including CAV1, ITGA5,
6 ITGB1, SNAI2, and TFDP1, was associated with poor clinical outcomes (Table 2 and
7 3). These clinical findings indicate that ARGs in HNSCC serve as important prognostic
8 indicators. To validate this observation, we used the HNSC-GSE65858 cohort, as
9 proposed in previous studies and confirmed using TCGA data, and found that elevated
10 anoikis resistance genes (ANRGs) expression was significantly associated with reduced
11 overall and disease-free survival (Supplementary Fig. 2A–B) [11].

12 **Development of anoikis-resistant HNSCC cells acquired spheroid formation and** 13 **a reduction in cell death**

14 To further investigate anoikis resistance signatures and associated molecular
15 mechanisms in head and neck cancer cells upon loss of extracellular matrix (ECM)
16 attachment, we cultured various head and neck cancer cell lines derived from different
17 anatomical sites (Table 4) under suspension conditions. During the 48-hour suspension
18 culture, we observed that most cell lines, except HONE-1 cells, formed tumor spheroids
19 rather than cell aggregates (Supplementary Fig. 3A).

20 We subsequently established anoikis-resistant (AR) HNSCC cell lines by
21 subjecting parental wild-type (WT) cells to repeated cycles of suspension and adherent
22 culture with slight modifications (Fig. 1A) [21, 35]. During suspension culture, we
23 observed that both WT and AR cells formed compact, well-defined spheroids at early
24 time points. However, as the culture period progressed, WT spheroids gradually
25 exhibited spheroid shrinkage and the accumulation of irregular debris adhering to the
26 spheroid periphery (Fig. 1B). Analysis of spheroid diameter revealed that under long-

1 term suspension culture conditions, compared with the WT group, the AR spheroid
2 group maintained its size more effectively and exhibited greater number of spheroids
3 (Fig. 1C). To determine whether the difference in spheroid size was attributable to
4 apoptosis, we collected spheroids formed by WT and AR of TU183 and HSC-3 cells
5 under suspension culture conditions. After the spheroids were dissociated into single
6 cells, apoptosis was analyzed by flow cytometry using Annexin V and PI staining.
7 Compared with those in WT spheroids, the proportion of apoptotic cells in AR
8 spheroids was significantly lower (Fig. 1D-F). Furthermore, live/dead cell
9 immunofluorescence assays also revealed a marked reduction in dead cells surrounding
10 the AR spheroids (Fig. 1G). Interestingly, as the number of selection cycles increased,
11 the number of apoptotic AR spheroids progressively decreased following suspension
12 culture (Supplementary Fig. 3B).

13 **ROS homeostasis is essential for AR survival under suspension culture conditions**

14 Cellular stress responses are frequently accompanied by excessive reactive oxygen
15 species (ROS) generation, resulting in oxidative stress that predisposes cells to
16 apoptosis [36, 37]. However, the precise role of ROS production in the regulation of
17 anoikis remains controversial and has yet to be fully elucidated. To investigate whether
18 the apoptosis observed in wild-type (WT) cells in the suspension culture was driven by
19 aberrant ROS accumulation, we employed a MitoSOX assay to specifically evaluate
20 mitochondrial superoxide production in WT-attached cells and spheroids. The results
21 revealed that compared with their adherent counterparts, WT spheroids not only
22 displayed a pronounced increase in mitochondrial ROS but also significantly exceeded
23 the levels measured in AR spheroids (Fig. 2A). Notably, WT cells exhibited sustained
24 ROS augmentation in a time-dependent manner during suspension, a trend that was
25 significantly abrogated in AR spheroids (Fig. 2B, Supplementary Fig. 4A). These

1 results suggest that AR spheroids may efficiently maintain ROS homeostasis, thus
2 avoiding anoikis upon detachment from the ECM.

3 To further confirm that ROS homeostasis is associated with anoikis resistance, we
4 treated WT cells with the ROS scavenger N-acetylcysteine (NAC) during suspension
5 culture. As shown in Fig. 2C and D, NAC treatment significantly reduced mitochondrial
6 ROS production and enhanced the survival of WT cells. Conversely, the administration
7 of FCCP and rotenone triggered the production of mitochondrial ROS in AR cells,
8 which ultimately reduced the cell viability of the AR spheroids (Fig. 2E, Supplementary
9 Fig. 4B). Concurrently, the pharmacological induction of ROS using the mitochondrial
10 OXPHOS inhibitor metformin also elicited a significant increase in the percentage of
11 apoptotic AR spheroids (Supplementary Fig. 4C and D). These findings indicate that
12 ROS homeostasis plays a pivotal role in mediating the survival of HNSCC spheroids.

13 **PTX3 is upregulated in anoikis-resistant HNSCC cells**

14 To further elucidate the molecular mechanisms underlying the acquisition of
15 anoikis resistance in AR cells to survive in the circulation system, we harvested both
16 WT and AR cells under adherent and suspension culture conditions and analyzed their
17 gene expression signatures using next-generation sequencing (NGS) (Fig. 3A). Gene
18 set enrichment analysis (GSEA) was performed to identify the top 10 enriched gene
19 sets in AR spheroids compared with those in WT spheroids. AR cells exhibited
20 increased expression of genes associated with cell cycle progression, cell growth, and,
21 notably, the EMT signature under suspension conditions (Fig. 3B and C). Consistent
22 with these findings, the expression of candidate genes related to anoikis resistance but
23 not the cell cycle was significantly upregulated in tumor samples, with the highest
24 expression observed in patients with the mesenchymal molecular subtype
25 (Supplementary Fig. 5A and B). Correlation studies also demonstrated that EMT

signatures were strongly associated with the expression of AR markers, suggesting that EMT may be associated with anoikis resistance in HNSCC (Supplementary Fig. 5C).

To identify the factors involved in the regulation of EMT and anoikis resistance, we performed an intersectional analysis of changes in the expression of gene related to EMT between WT and AR cells under both adherent and suspension conditions. Through this screening process, 17 commonly overexpressed EMT-related genes were identified by intersecting the 60 and 34 highly expressed EMT markers in AR-compared with WT-attached cells and in AR compared with WT spheroids, respectively (Fig. 3D and Supplementary Fig. 6A and B). Correlation analysis revealed that these 17 genes were strongly correlated with anoikis resistance genes in the HNSC-GSE65858 dataset and were more predominantly expressed in tissues from patients classified as mesenchymal (Supplementary Fig. 6C and D). In addition, functional protein association network analysis using the STRING database revealed two major gene clusters among the 17 genes, primarily involving extracellular remodeling and cytokine signaling (Fig. 3E), which were also to be evaluated for their prognostic value in HNSCC patients (Table 5). These results suggest that the expression of cytokine signaling-associated molecules, particularly PTX3, is significantly upregulated in tumor tissues and is associated with poor clinical outcomes. Consistent with these results, PTX3 expression levels were significantly higher in AR cells than in WT cells in both adherent and suspension cultures (Fig. 3F-H).

To further examine whether increased PTX3 is specifically expressed in suspended HNSCC cells, different cancer cells in suspension-related culture models from the Gene Expression Omnibus (GEO) database were analyzed. Notably, upregulation of PTX3 expression was observed exclusively in HNSCC spheroid models, but not in other cancer types (Supplementary Fig. 7A). Consistently, increased PTX3

expression was also observed in various suspended HNSCC cell lines (Supplementary Fig. 7B and C).

The upregulation of PTX3 expression facilitates anoikis resistance and enhances metastatic potential

We further investigated whether PTX3 expression is related to AR survival and metastasis. First, we analyzed the impact of PTX3 on HNSCC outcomes. As shown in Figure 4A, stratification by PTX3 expression cutoff values revealed that higher PTX3 expression was associated with poorer clinical outcomes among patients with advanced-stage head and neck cancer. To study whether PTX3 expression regulates AR spheroid formation, PTX3 was silenced in cells by using small interfering RNA (siRNA). The results revealed that PTX3 depletion markedly reduced the spheroid diameter and increased the apoptotic population in AR spheroids (Fig. 4B-C). To verify whether PTX3-regulated spheroid survival is mediated by the inhibitor of apoptosis (IAP) family, we first constructed a heatmap of IAP members using DEGs from RNA-seq data (Fig. 3A) and found that the expression of BIRC5 (survivin) was consistent with the cell survival rate (Supplementary Fig. 8A). PTX3 depletion subsequently significantly inhibited the expressions of survivin and AKT phosphorylation (Thr308) (Fig. 4D). Concurrently, the DepMap database also revealed lower expression of survivin in HNSCC cell lines subjected to shRNA-mediated PTX3 silencing (Supplementary Fig. 8B). Furthermore, survivin inhibition using survivin siRNA and the inhibitor YM155 significantly increased the apoptosis rate of suspended AR sphere cells (Supplementary Fig. 8C and D). Collectively, these results indicate that the PTX3/survivin axis is essential for the survival of suspended AR cells.

We further analyzed the impact of PTX3 on HNSCC metastasis. Clinical datasets from the TCGA and GEO databases revealed that PTX3 expression was significantly elevated in mesenchymal and N1-stage tumor tissues with lymph node metastasis

1 (Supplementary Fig. 9A-B). Furthermore, correlation analysis revealed that high PTX3
2 expression was associated with EMT marker expression in both clinical specimens and
3 HNSCC cell lines from the DepMap database (Supplementary Fig. 9C–D). Moreover,
4 we analyzed PTX3-positively correlated genes and found that they were significantly
5 enriched in pathways related to the cytoskeleton, extracellular matrix (ECM)
6 organization, and focal adhesion (Table 6). These findings prompted us to investigate
7 whether PTX3 contributes to the metastatic potential of AR cells.

8 On the basis of the finding that AR spheroids strongly expressed EMT signatures
9 (Fig. 3B, Supplementary Fig. 5), we further confirmed the higher expression of EMT
10 markers in suspended AR cells than in suspended WT cells (Fig. 5A-B). Furthermore,
11 conditioned medium (CM) collected from AR spheroid cultures substantially increased
12 MMP activity (Fig. 5C). Intriguingly, compared with WT cells, AR cells exhibited
13 distinct morphological changes and enhanced motility features upon reattachment,
14 including increased levels of phosphorylated FAK and an F-actin cytoskeleton (Fig.
15 5D). These results suggest that AR cells acquire stronger metastatic ability. To assess
16 whether PTX3 expression regulates the metastatic potential of AR cells, we used siRNA
17 to silence PTX3 in AR cells. The results revealed that PTX3 depletion inhibited VIM
18 and MMP expression, as well as MMP activity, in AR cells (Fig. 5E and Supplementary
19 Fig. 10). In addition, PTX3 silencing effectively suppressed the migratory capacity of
20 AR cells (Fig. 5F-G). Metastatic colonization requires cancer cells to interact with the
21 vascular endothelium during circulation. Therefore, AR cell adhesion to endothelial
22 cells was analyzed. As shown in Fig. 6A, compared with parental WT cells, AR cells
23 exhibited a greater capacity for adhesion to endothelial cells, and this adhesion was
24 significantly inhibited by PTX3 knockdown. In addition, an *in vivo* animal study
25 revealed that compared with WT cells, AR cells exhibited greater extravasation and
26 lung tumor nodule formation, and these effects were inhibited under PTX3 knockdown

conditions (Fig. 6B-C). Collectively, these results indicate that PTX3 plays an essential role in promoting metastatic competence in anoikis-resistant HNSCC cells.

Autophagy homeostasis triggers PTX3 expression through ATG5 and ATG12 to promote the survival of AR spheroids

Emerging evidence suggests that detached cancer cells activate autophagy to generate the energy required for survival [22, 38]. To determine whether autophagy is differentially activated to promote the survival of suspended AR spheroids, we quantified autophagic activity using CYTO-ID staining. The results revealed massive autophagic activation in WT spheroids compared with that in adherent cells, whereas this activation was significantly reduced in AR spheroids (Supplementary Fig. 11).

To investigate the correlation between autophagic balance and the viability of suspended AR cells, we targeted different phases of the autophagic process. We used 3-MA to block autophagosome initiation and Baf-A1 or CQ to prevent lysosomal degradation. On the other hand, we also used rapamycin to induce excessive autophagic flux in AR spheroids. Interestingly, the results revealed that disrupting autophagic homeostasis, either by inhibition or overactivation, significantly reduced the survival rate of AR spheroids (Fig. 7A, Supplementary Fig. 12). These findings indicate that autophagic homeostasis is a critical determinant of anoikis resistance. Interestingly, the expressions of PTX3 and LC3B were reduced in 3-MA but not Baf-A1 or CQ-treated AR spheroids (Fig. 7B). In addition, treatments with various canonical inhibitors, including those against NF- κ B, JNK, PI3K/AKT, ERK, and FAK, demonstrated that inhibition of PI3K/AKT expression maximally suppressed PTX3 expression in AR spheroids (Fig. 7C). These results indicate that the expression of PTX3 is associated with the early initiation of autophagy. Early formation of the autophagosome is intrinsically linked to ATG family proteins. Therefore, we utilized siRNA to target ATG5, 7, 12, and 16, which are essential for autophagosome assembly. The results

1 revealed that silencing ATG5 and ATG12 expression significantly reduced PTX3
2 expression in AR spheroids (Fig. 7D). To further investigate whether the expression of
3 PTX3 and its upstream and downstream factors, such as ATG5/12 and survivin, is
4 associated with HNSCC outcome, we analyzed the correlation between patient survival
5 and the expression of ATG5, ATG12, PTX3, and survivin in the mesenchymal subtype.
6 Although survivin expression was significantly associated with reduced overall
7 survival, the other genes were not significantly associated with disease-free survival
8 (Fig. 7E). Interestingly, the integrated expressions of genes such as
9 ATG5/ATG12/PTX3/survivin were significantly associated with poor overall survival
10 and disease-free survival, highlighting the potential of this molecular signature as a
11 prognostic predictor for patients with metastatic HNSCC (Fig. 7E).

12 **ROS-mediated EGFR activation maintains the protective autophagy signaling in** 13 **AR spheroids**

14 Previous studies have shown that anchorage-independent stress triggers autophagy
15 and antioxidant responses as survival mechanisms [39]. To further characterize whether
16 ROS homeostasis is associated with autophagic adaptation in suspended AR cells, we
17 analyzed ROS accumulation and autophagic activity. First, we found that NAC-
18 mediated ROS depletion reduced autophagic activity in suspended WT cells
19 (Supplementary Fig. 13A), which was consistent with the reduction in apoptosis in
20 NAC-treated suspended WT cells (Fig. 2C). Conversely, disruption of ROS
21 homeostasis using FCCP and rotenone in suspended AR cells not only triggered
22 excessive ROS levels but also dysregulated autophagic activity (Fig. 2E and
23 Supplementary Fig. 13B). These results suggest that maintaining ROS homeostasis is
24 essential for suspended cell survival through the modulation of autophagic activity.

25 Previous studies have highlighted the interplay between EGFR signaling and
26 reactive oxygen species (ROS) in cancer cells [21]. Notably, stress-induced autophagic

1 activation has also been shown to be modulated by EGFR signaling. [40-42]. To verify
2 whether ROS-mediated protective autophagy enhances suspended AR cell survival via
3 EGFR activation, the phosphorylation of EGFR, FAK, ERK, and BCL2 in AR
4 spheroids was examined. As shown in Supplementary Fig. 13C, increased EGFR
5 phosphorylation was observed in suspended AR but not WT cells. Conversely, the
6 phosphorylation of FAK, ERK, and BCL2 decreased in suspended culture. In addition,
7 disruption of ROS homeostasis inhibited EGFR expression and activation,
8 accompanied by a reduction in the expression of the protective autophagy marker p62
9 and downregulation of survivin expression in suspended AR cells (Supplementary Fig.
10 13D). Consistently, inhibition of EGFR activation using TKIs, such as gefitinib and
11 erlotinib, also inhibited p62 expression, and increased autophagic activity, followed by
12 reduced survivin levels and increased apoptosis in suspended AR cells (Supplementary
13 Fig. 14). These results suggest that EGFR activation by appropriate ROS levels is
14 essential for maintaining protective autophagy to increase the survival of AR spheroids.

15 **PTX3 glycosylation enhances anoikis resistance in head and neck cancer.**

16 Interestingly, we found that the PTX3 protein appeared as two distinct forms under
17 suspension culture conditions (Figs. 3H, 7B-D, 8A, and Supplementary Fig. 7B).
18 According to GeneCards (<https://www.genecards.org/>), PTX3 is predicted to undergo
19 glycosylation and phosphorylation. To clarify whether the higher molecular weight of
20 PTX3 is due to post-translational modifications, such as phosphorylation or
21 glycosylation, cell lysates from suspension cultures were treated with calf intestinal
22 alkaline phosphatase (CIP) to remove phosphorylation or PNGase F to cleave N-linked
23 oligosaccharides. As shown in Fig. 8B and C, PNGaseF, but not CIP, significantly
24 removed the higher-molecular-weight protein. Consistently, treatment of suspended
25 AR cells with oligosaccharyltransferase and N-linked glycosylation inhibitors, such as
26 NGI-1 and tunicamycin, respectively, reduced the abundance of the higher PTX3 band

1 (Fig. 8D). These results suggest that PTX3 undergoes N-linked glycosylation
2 specifically under suspension culture conditions.

3 Previous reports have indicated that PTX3 can be glycosylated at Asn220 [30]. To
4 investigate whether glycosylated PTX3 is associated with the survival of suspended AR
5 cells, we constructed a glycosylation-deficient mutant expression vector harboring an
6 N220A substitution, and the results further suggest that the higher molecular weight of
7 PTX3 was due to glycosylation at Asn220 during suspension (Fig. 8E). Functional
8 assays revealed that treatment with glycosylation inhibitors, either tunicamycin or NGI-
9 1, as well as the expression of the PTX3-N220A mutation in AR cells, significantly
10 impaired spheroid formation, thus reducing AR cell survival (Fig. 8F-H). These results
11 indicate that protein glycosylation may be essential for the survival of cancer cells in
12 suspension. Indeed, we found that tunicamycin significantly inhibited survivin
13 expression in suspended AR cells (Supplementary Fig. 15A and B). Consistently,
14 disruption of protein glycosylation also triggered mitochondrial ROS generation and
15 excessive autophagic activation in AR spheroids (Supplementary Fig. 15C and D).
16 These results highlight that protein glycosylation, such as PTX3 glycosylation, is
17 crucial for conferring anoikis resistance in HNSCC.

Discussion

In the treatment of head and neck cancer, advancements in therapeutic approaches, including the introduction of immune checkpoint inhibitors, have led to significant improvements in overall patient survival [2, 43]. Nevertheless, the mortality associated with cancer metastasis remains unacceptably high [7]. In this study, through the selection of anoikis-resistant cell lines, we identified a critical role for PTX3 in promoting spheroid survival and metastasis in head and neck cancer. Mechanistically, we propose a novel regulatory mechanism involving homeostatic ROS-induced activation of autophagy to control PTX3 expression and post-translational modification. These findings provide a foundation for developing innovative therapeutic strategies by targeting PTX3/survivin for metastatic head and neck cancer.

Current research remains divided into the mechanisms by which ROS influence the survival of detached cells. For example, a study on anoikis resistance in gastric cancer revealed that cells increase ROS via activation of NADPH oxidase 4 (NOX4), which subsequently upregulates EGFR expression and enhances cell survival during suspension [21]. Similarly, a report on breast cancer indicated that fluid shear stress triggers ROS and nitric oxide (NO) signaling to maintain caveolin-1 expression, thus conferring anoikis resistance [44]. However, cancer cells disseminating into the vasculature sustain cell survival through ATF4-mediated heme oxygenase-1 (HO-1) expression to alleviate oxidative stress [39]. Interestingly, our study revealed that excessive ROS accumulation induced cell death in WT spheroids, but AR spheroids maintained balanced ROS levels, thereby preserving their survival. Conversely, inducing an overproduction of ROS in AR spheroids effectively drives apoptosis. These findings underscore that maintaining oxidative stress homeostasis is essential for suspended cell survival.

On the other hand, numerous studies have established that the induction of

1 autophagy is indispensable for the survival of cells in suspension culture [22, 38, 39].
2 In this study, we also provide evidence to show that the survival of AR spheroids
3 depended on ROS-regulated autophagic activation, suggesting that ROS serve as a
4 primary trigger for the autophagic response. Activation of autophagy can clear damaged
5 organelles and toxic proteins, functioning as a protective mechanism (protective
6 autophagy) that helps tumor cells survive acute stress and maintain their metastatic
7 potential [38, 45]. Mechanistically, the interplay between autophagy and EGFR serves
8 as a critical pivot point that determines cancer cell adaptation to adverse conditions and
9 survival. For example, activated EGFR robustly stimulates the downstream PI3K/AKT
10 cascade, which subsequently activates the mammalian target of rapamycin (mTOR) to
11 inhibit autophagy [46]. Moreover, when cancer cells encounter hypoxia or detachment
12 conditions, these conditions promote EGFR activation and inhibit overactive autophagy,
13 resulting in sustained cancer cell survival [22, 47]. Studies also indicate that under
14 cellular stress, such as starvation, EGFR promotes cancer cell survival through a kinase-
15 independent mechanism that directly initiates the autophagy machinery [48, 49].
16 Although EGFR activation may either induce or inhibit autophagy depending on
17 various cellular statuses, these results suggest that EGFR activation affects cell survival
18 by modulating autophagy in response to cellular stress, even under suspended
19 conditions [21, 22]. Indeed, our study shows that in controlling ROS levels of
20 suspended AR cells, EGFR activation significantly triggered p62 expression, leading
21 to cell survival through maintaining protective autophagy. Collectively, these results
22 reveal the essential role of the ROS/EGFR/autophagy axis in regulating the survival of
23 spheroid cells.

24 As a key protein that is responsive to environmental stimuli and cellular stress,
25 PTX3 has been implicated in redox regulation [50, 51]. In this study, we revealed that
26 PTX3 expression was significantly upregulated upon activation of the

1 ROS/EGFR/autophagy axis. Intriguingly, PTX3 expression strongly correlated with the
2 protein levels of ATG5, ATG12, and LC3B. We also found that depletion of ATG5 and
3 ATG12 significantly inhibited PTX3 gene and protein expression, suggesting that
4 ATGs not only regulate autophagosome formation but also mediate gene expression,
5 including PTX3. Consistent with our findings, in addition to its canonical role in
6 regulating autophagy, the ATG5-12 complex performs autophagy-independent
7 functions, including the modulation of gene transcription [52]. In addition, previous
8 studies have shown that miR-103a influences HCV replication via an ATG5-dependent
9 pathway [50] and that the ATG5-12 axis regulates oxidative stress in melanocytes [51].
10 ATG5-12 complex also interacts with RNA-binding proteins and transcription factors
11 to regulate mRNA stability or translational efficiency [53, 54]. These results suggest
12 that ATGs confer cancer cellular functions, such as correlation with gene expression,
13 in addition to orchestrating autophagosome formation.

14 Emerging evidence indicates that cancer cells upregulate BIRC5/survivin
15 expression to mitigate excessive autophagy and preserve DNA integrity. [55, 56]. PTX3
16 has been reported to modulate survivin expression in bovine luteinized granulosa cells
17 to regulate cell viability [57]. However, its contribution to the metastatic cascade and
18 spheroid survival has not yet been fully elucidated. We further investigated whether
19 survivin is essential for ATG-induced PTX3 to regulate survival of suspended AR cells.
20 For the first time, we provide evidence that PTX3 regulates survivin expression to
21 maintain AR spheroid survival in head and neck cancer. Concurrently, disruption of
22 protective autophagy by treating AR spheroids with either ROS inducers or EGFR TKIs
23 significantly downregulated survivin expression, thus decreasing spheroid viability.
24 These results indicate that the activation of the protective autophagy/PTX3/survivin
25 axis is essential for AR spheroid survival. We also further confirmed that the combined
26 expression signature of ATG5, ATG12, PTX3, and survivin serves as a robust

1 prognostic indicator for survival in patients with metastatic head and neck cancer. These
2 results offer novel insights into the future clinical assessment of circulating tumor cells.

3 The preservation of glycosylation is a vital step for many proteins to perform their
4 biological functions. For instance, in breast cancer models, disrupting the N-linked
5 glycosylation of EpCAM induces autophagy-dependent apoptosis [58]. Furthermore,
6 glycosylation plays a pivotal role in drug resistance and in ligand-receptor binding
7 affinity. Interestingly, an increasing number of studies have highlighted the importance
8 of PTX3 glycosylation. For example, glycosylated PTX3 is involved in complement
9 activation and in neutralizing viral toxicity [59, 60]. However, the specific role of PTX3
10 glycosylation in cancer progression remains poorly understood. Given that previous
11 literature highlights the involvement of glycosylated PTX3 in several cellular processes
12 [50, 61, 62], we further explored the functional role of PTX3 glycosylation in HNSCC
13 spheroids. We observed distinct patterns of PTX3 expression and increased PTX3
14 glycosylation upon detachment, both of which are essential for spheroid viability.
15 Notably, maintaining PTX3 glycosylation during the suspension phase is also involved
16 in survivin expression. Disruption of glycosylation in AR spheroids significantly
17 reduced survivin expression, concomitantly promoting ROS accumulation and
18 excessive autophagic activation, ultimately leading to the death of tumor spheroids.
19 Consequently, these results support the notion that targeting glycosylation, such as
20 PTX3 glycosylation, during the metastatic cascade of head and neck cancer may affect
21 therapeutic efficacy.

22 Taken together, the results of our systematic analysis of WT and AR spheroids in
23 suspension cultures elucidate a pivotal mechanism through which the control of
24 oxidative stress-regulated protective autophagy affects cell survival via the activation
25 of the PTX3/survivin axis. Crucially, ligand-independent EGFR activation is
26 indispensable for driving this protective autophagy in spheroids. The protective

1 autophagy maintains cell viability through the ATGs-regulated PTX3/survivin axis in
2 suspended AR cells. These results demonstrate for the first time that the dynamic
3 glycosylation of PTX3 is vital for spheroid viability during metastasis, highlighting that
4 glycosylated PTX3 is a promising therapeutic target. Ultimately, the novel ATG5-
5 12/PTX3/survivin axis proposed herein offers a new diagnostic marker for evaluating
6 the progression of metastatic HNSCC.

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1 **Declarations**

2 **Ethics approval and consent to participate**

3 All animals were sourced from the National Cheng Kung University (NCKU)
4 Laboratory Animal Center (Tainan, Taiwan), and all procedures were approved by the
5 Institutional Animal Care and Use Committee (IACUC) of NCKU Medical College
6 (Approval No. NCKU-IACUC-113-207).

7

8 **Consent for publication**

9 Not Applicable

10

11 **Availability of data and materials**

12 The data that support the findings of this study are available in the methods and/or
13 supplementary material of this article.

14

15 **Competing interests**

16 The authors declare no competing interests.

17

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21

22 **Authors' contributions**

23 In this chapter, all authors are referred to by their initials. (Ting-Wei Chang: TWC,
24 Wan-Chen Huang: WCH, Shih-Hung Chan: SHC, Jhih-Peng Tsai: JPT, Jing-Ting
25 Chiou: JTC, Hsiao-Yu Chang: HYC, Ying-Zhu Meow: YZM, Tsan-Yu Chen: TYC,
26 Ju-Ming Wang: JMW, Bing-Han Chung: BHC, Wen-Chang Chang: WCC and Ben-

1 Kuen Chen: BKC). TWC, WCC, and BKC devised the project, the main conceptual
2 ideas, proof outline, processed the experimental data, and wrote the manuscript. WCH,
3 SHC, JMW and BHC verified the analytical methods, derived the models, and analyzed
4 the data. TWC, JPC, JTC, HYC, YZM and TYC performed the experiments and
5 processed the experimental data. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Establishment of anoikis-resistant (AR) HNSCC cells. (A) Schematic of establishing AR-TU183 cells from wild-type (WT) cells under slightly modified adhesion and suspension cycles. (B-C) WT and AR cells were cultured in Ultra-low attachment culture dish. Tumor spheroids were taken pictures with brightfield as indicated time point. The diameter of WT and AR spheroids were measured on day 7 using ImageJ software. (D-E) TU183 WT/ AR cells were cultured under suspension for 7 days. Tumor spheroids were harvest and dissociated into single cells by accumax reagent. Cells were then stained with annexin V and Propidium Iodide (PI) to measure apoptosis by flow cytometry (left panel). The percentage of apoptotic cells were statistically analyzed by Prism 6.0 software (right panel). (F) HSC-3 WT/ AR cells were cultured under suspension for 7 days. Tumor spheroids were harvest and dissociated into single cells by accumax reagent. Cells were then stained with annexin V and Propidium Iodide (PI) to measure apoptosis by flow cytometry. The percentage of apoptotic cells were statistically analyzed by Prism 6.0 software. (G) WT and AR cells were cultured under suspension for 7 days. Tumor spheroids were then stained with Calcein-AM and PI to detect the live and dead cells using fluorescent microscopy. Scale bar determined 200 μm in regular image and 20 μm in zoom in image.

Figure 2. Manipulation of intracellular ROS levels affects the WT and AR spheroids survival under suspension culture. (A) Adherent WT cells and WT/ AR spheroids were cultured under suspension for 2 days. Tumor spheroids were harvest and dissociated into single cells by accumax reagent. Cells were then stained with annexin V and Propidium Iodide (PI) to measure apoptosis by flow cytometry. (B) Adherent WT cells and WT spheroids were cultured under suspension for indicated days. Tumor spheroids were harvest and dissociated into single cells by accumax reagent. Cells were then

1 stained with mitoSOX to measure mitochondrial ROS by flow cytometry. (C) WT
2 spheroids were cultured with/ without 10 mM NAC under suspension for 7 days. Tumor
3 spheroids were harvest and dissociated into single cells by accumax reagent. Cells were
4 then stained with annexin V and Propidium Iodide (PI) to measure apoptosis by flow
5 cytometry (left panel). The percentage of apoptotic cells were statistically analyzed by
6 Prism 6.0 software (right panel). (D) WT spheroids were cultured with/ without 10 mM
7 NAC under suspension for 7 days. Tumor spheroids were harvest and dissociated into
8 single cells by accumax reagent. Cells were then stained with MitoSOX to measure
9 mitochondrial ROS by flow cytometry. (E) AR spheroids were cultured with/ without
10 10 μ M FCCP or 1 μ M rotenone under suspension for 7 days. Tumor spheroids were
11 harvest and dissociated into single cells by accumax reagent. Cells were then stained
12 with annexin V and Propidium Iodide (PI) to measure apoptosis by flow cytometry (left
13 panel). The percentage of apoptotic cells were statistically analyzed by Prism 6.0
14 software (right panel).

15

16 Figure 3. GSEA analysis in AR cells revealed potential targets facilitaed in anoikis
17 resistance and epitheial-mesenchymal transition. (A) Schematic diagram of the process
18 of RNA sequencing from both WT/ AR attached cells and spheroids. (B) Gene set
19 enrichment analysis of gene expression profiles from WT and AR spheroids.
20 Expression data from AR spheroids showed the top 15 with the most significant altered
21 compared to WT spheroids. (C) GSEA for the gene signature of the epithelial-
22 mesenchymal transition. (D) Overlapping of alterd gene expression between AR
23 adherent cell and spheroids. (E) Functional protein association networks by STRING
24 analysis of the 17 altered genes in both AR adherent cells and spheroids. (F) The
25 normalized counts of the PTX3 expression from the raw-read sequence data between
26 WT/ AR attached cells and spheroids. (G-H) WT/ AR attached cells and spheroids were

1 cultured for 2 days; cells and spheroids were harvested and detected mRNA and protein
2 expression of PTX3 and GAPDH by using RT-qPCR and western blotting.

3
4 Figure 4. The expression of PTX3 is associated with HNSCC progression and sustained
5 spheroids survival. (A) Kaplan-Meier curves showed HNSCC patient overall survival
6 that was retrieved from the TCGA database (n=430, including 94 patients within stage
7 I, II and 336 patients within stage III and IV by cut-off the expression of PTX3 with
8 median, tertile and quartile). (B) WT and AR cells were transfected with 40 nM PTX3
9 siRNA or scrambled (SC) oligonucleotides for 24 h. Cells were then cultured under
10 suspension for 7 days. Tumor spheroids were taken pictures with brightfield and
11 measured the diameter. (C) WT and AR cells were transfected with 40 nM PTX3
12 siRNA or scrambled (SC) oligonucleotides for 24 h. Cells were then cultured under
13 suspension for 7 days. Tumor spheroids were harvest and dissociated into single cells by
14 accumax reagent. Cells were then stained with annexin V and Propidium Iodide (PI) to
15 measure apoptosis by flow cytometry (i). The percentage of apoptotic cells were
16 statistically analyzed by Prism 6.0 software (ii). (D) WT and AR cells were transfected
17 with 40 nM PTX3 siRNA or scrambled (SC) oligonucleotides for 24 h. Cells were then
18 cultured under suspension for 2 days. The protein expressions of PTX3, survivin, p-AKT
19 and GAPDH were examined using western blotting.

20
21 Figure 5. Up-regulation of PTX3 in AR cells promotes migration and invasion through
22 down-stream vimentin and MMPs expression. (A) The normalized counts of CDH1,
23 CDH2, VIM, MMP-3 and MMP9 from the raw-read sequence data among WT adherent
24 cells and WT/AR spheroids. (B) WT adherent cells, WT spheroids and AR spheroids
25 were cultured for 2 days, CDH1, CDH2, VIM, MMP-3 and MMP9 and GAPDH
26 expression were examined using RT-qPCR. (C) WT adherent cells, WT spheroids and

1 AR spheroids were cultured for 2 days. The condition medium was harvested for
2 analyzing MMP activity as described in the materials and methods. (D) WT and AR
3 cells were cultured on μ -Slide 4 Well coverslips for 2 days. Cells were then stained
4 with phalloidin, red fluorescent -conjugated p-FAK antibody and DAPI. The
5 immunofluorescence image was taken by confocal microscopy. (E) WT and AR cells
6 were transfected with 40 nM PTX3 siRNA or scrambled (SC) oligonucleotides for 24 h.
7 Vimentin, MMP-3, -9 and GAPDH were examined using western blotting. (F-G) WT
8 and AR cells were transfected with 40 nM PTX3 siRNA or scrambled (SC)
9 oligonucleotides for 24 h. Migrating and invading cells were stained with crystal violet
10 and imaged with a microscope (lower panel) and then solubilized in 10% acetic acid
11 for quantification of migrating and invasive levels, as shown in columns (upper panel).
12 The absorbance was measured at a wavelength of 595 nm. Scale bar determined 20 μ m
13 in regular image and 5 μ m in zoom in image.

14
15 Figure 6. PTX3 is critical for tumor-endothelial interactions and in vivo metastasis. (A)
16 WT and AR cells were transfected with 40 nM PTX3 siRNA or scrambled (SC)
17 oligonucleotides for 24 h. The DiI-labeled tumor cells settled down on the monolayer
18 of endothelial cells for 30 minutes. The interacted tumor cells were measured by flow
19 cytometry (left panel) and the quantification were shown in columns (right panel). (B)
20 WT and AR Cells were transfected with 40 nM PTX3 siRNA or scrambled
21 oligonucleotides (SC) for 24 hours. Cells were then injected intravenously into the tail
22 vein of mice. At 48 hours after injection of tumor cells, the mice were sacrificed to
23 examine metastatic tumor cells surrounding the lung tissue as described in “Materials
24 and Methods.” Tumor cell penetration was imaged using a microscope (left panel).
25 Original magnification $\times 20$; DiI-labeled tumor cells (red); CD31-labeled blood vessels
26 (green); DAPI-labeled nucleus (blue). The number of tumor cell extravasation was

1 calculated by analyzing at least five sections and six fields (right panel). Dots indicate
2 the number of mice. (C) WT and AR Cells were transfected with 40 nM PTX3 siRNA
3 or scrambled oligonucleotides (SC) for 24 hours. Cells were then injected intravenously
4 into the tail vein of mice. To evaluate lung metastasis, mice were sacrificed up to
5 8 weeks after injection. H&E staining of lung tissue was imaged with the whole slide
6 screening using BX63 upright microscope (left panel). The number of micronodules
7 was counted under a microscope (right panel). Dots indicate the number of mice.

8

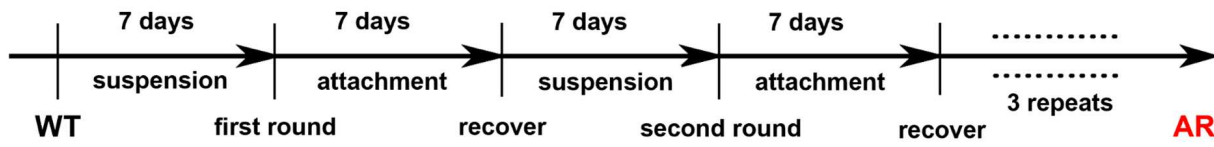
9 Figure 7. Early stage of autophagic activation collaboratively with PTX3 in regulating
10 HNSCC progression. (A) AR cells were cultured with or without different phases of
11 autophagy inhibitor including 3-MA, Baf-A1 and CQ under suspension for 7 days.
12 Tumor spheroids were taken pictures with brightfield and measured the diameter
13 followed by apoptosis detection assay. (B) AR cells were cultured with or without
14 canonical inhibitors including 5mM 3-MA, 100ng/ ml Baf-A1 and 20 mM CQ under
15 suspension for 7 days. The protein expressions of Beclin-1, LC3B, PTX3, p-BCL2 and
16 α -tubulin were examined using western blotting. (C) AR cells were cultured with or
17 without 10 μ M canonical inhibitors under suspension for 7 days. The protein
18 expressions of PTX3 and GAPDH were examined using western blotting. (D) AR cells
19 were transfected with 40 nM ATG5, ATG7, ATG12 and ATG16 siRNA or scrambled
20 oligonucleotides (SC) for 24 hours. Cells were then cultured under suspension for 2 days.
21 The protein expressions of PTX3, ATG5, ATG7, ATG12, ATG16L1 and GAPDH were
22 examined using western blotting. (E) Kaplan–Meier curves showed HNSCC patient
23 under mesenchymal subtype over-all and disease-free survival were retrieved from the
24 TCGA database (n = 74). The p-value of the Log-rank test and Hazard ratio (HR)
25 compared patients with high and low expression of ATG5, ATG12, BIRC5 (survivin),
26 and PTX3 by comparing their individual and collective expression levels that were

shown in the figures. 3-MA: 3-Methyladenine. Baf-A1: Bafilomycin A1. CQ: chloroquine. Par: parthenolide. SP: SP600125. LY: LY294002. U0: U0126.

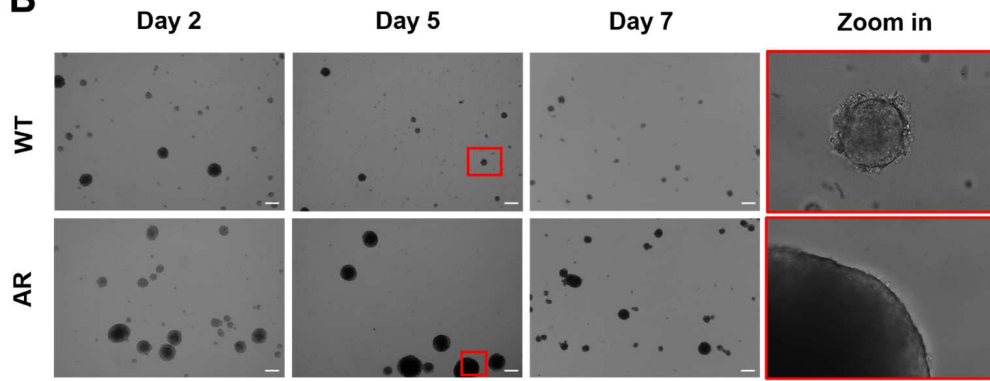
Figure 8. PTX3 sustained spheroids survival through glycosylated modification. (A) WT attached cells and spheroids were cultured for 2 days. Attached cells and spheroids were harvested and detected protein expressions of PTX3 and GAPDH by using western blotting. (B) WT cells were cultured under suspension for 2 days. Tumor spheroids were harvested for protein detection. Cell lysate was treated with Peptide-N-Glycosidase-F (PNGase F) for 2 hours followed by western blotting examining the expression of PTX3. (C) WT cells were cultured under suspension for 2 days. Tumor spheroids were harvested for protein detection. Cell lysate was treated with 10 units of calf intestinal alkaline phosphatase (CIP) for 30 minutes followed by western blotting examining the expression of PTX3 and GAPDH. (D) AR cells were cultured with or without 100ng/ ml TM or 10 μ M NGI-1 treatment for 2 days under suspension. The expressions of PTX3 and GAPDH were examined using western blotting. (E) AR cells were transfected with empty vector (EV), expression vector of PTX3, truncated 220A mutant for 24 hours. Cells were then cultured in ultra-low attachment plate for 2 days. The expressions of PTX3 and GAPDH of spheroids were examined using western blotting. (F-G) AR cells were cultured with or without TM or NGI-1 under suspension for 7 days. Tumor spheroids were harvest and dissociated into single cells by accumax reagent. Cells were then stained with annexin V and Propidium Iodide (PI) to measure apoptosis by flow cytometry. (H) AR cells were transfected with EV or PTX3-220A expression vector. Cells were then cultured under suspension for 7 days. Tumor spheroids were harvest and dissociated into single cells by accumax reagent. Cells were then stained with annexin V and Propidium Iodide (PI) to measure apoptosis by flow cytometry.

Figure 1

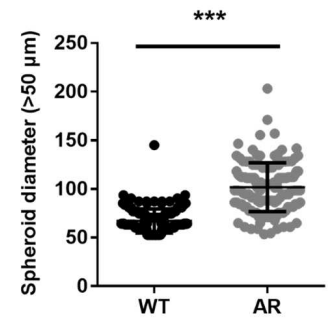
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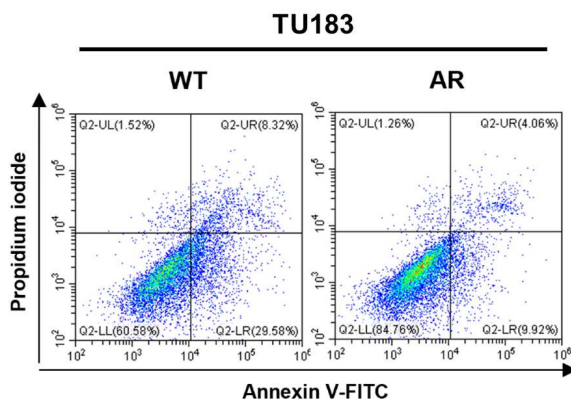
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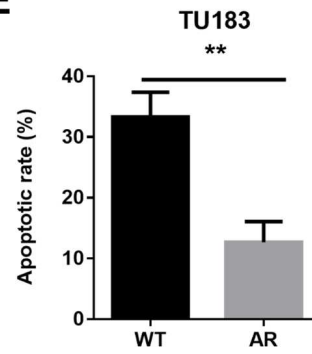
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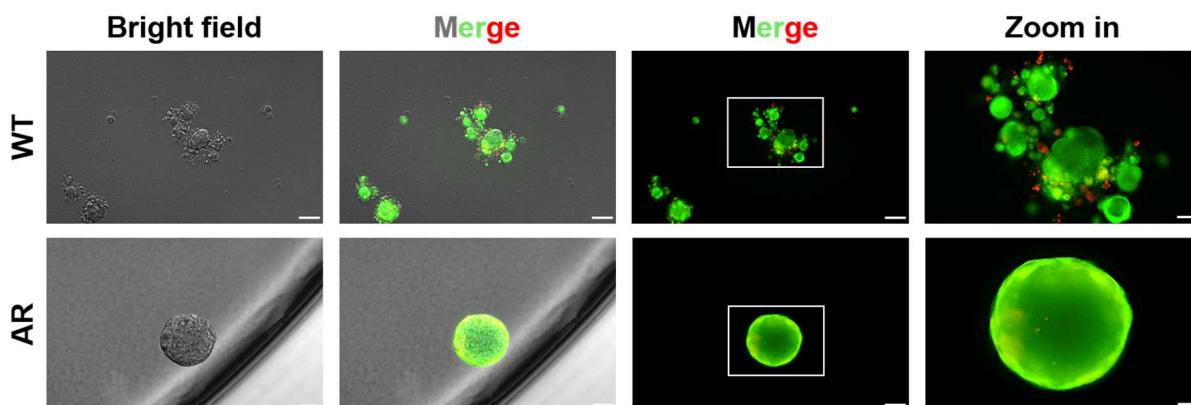
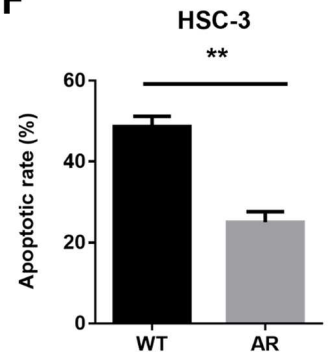


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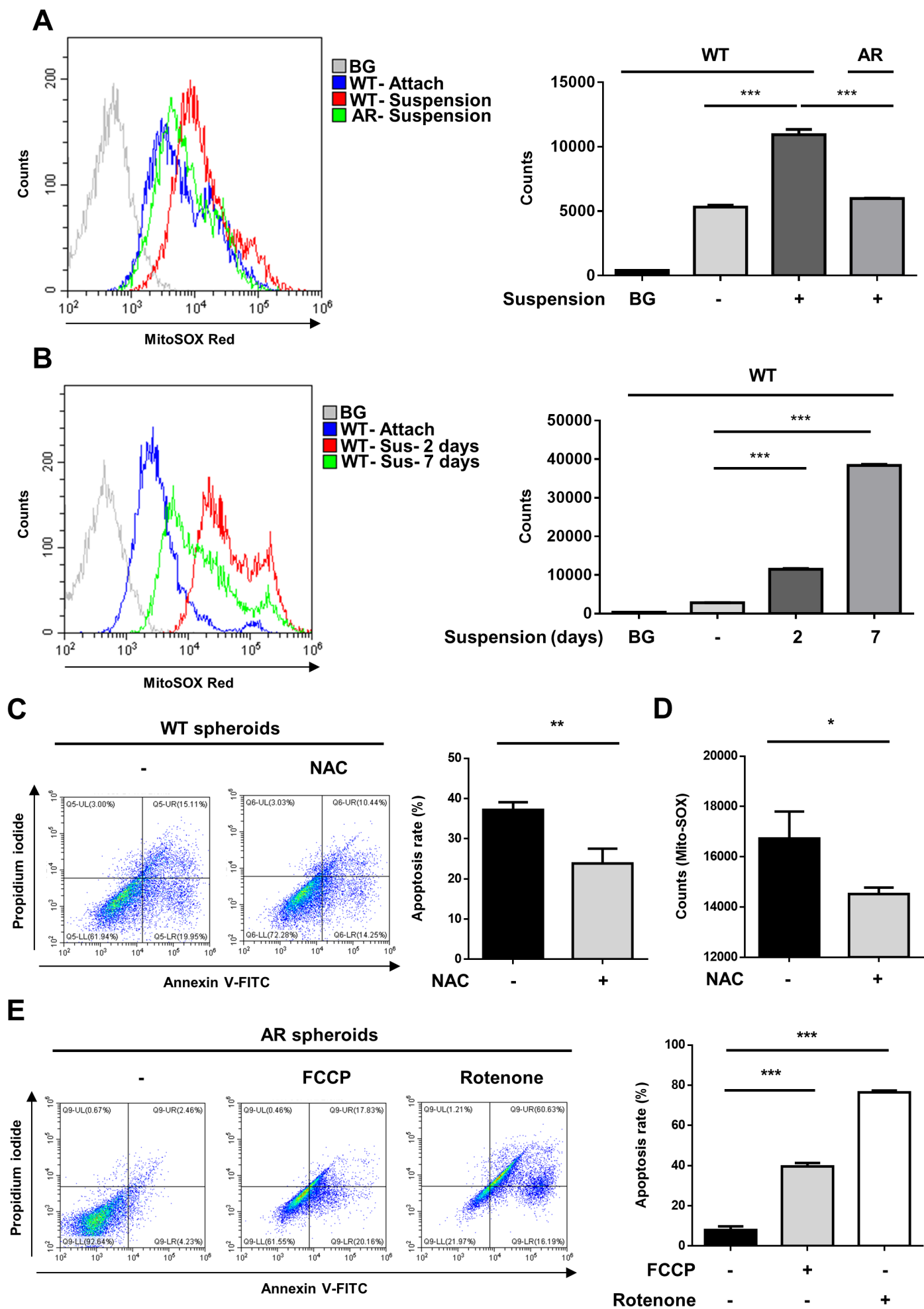


Figure 3

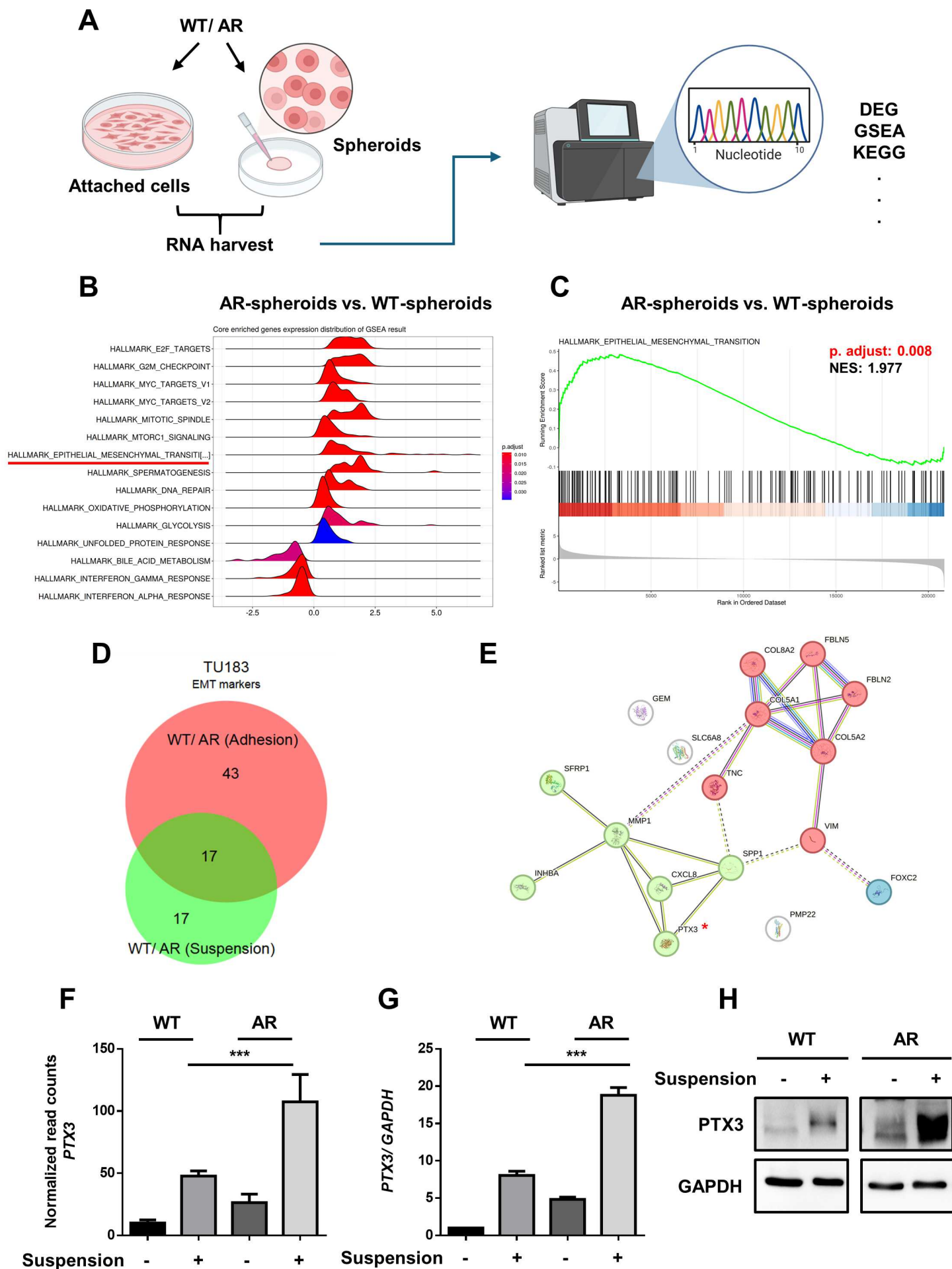
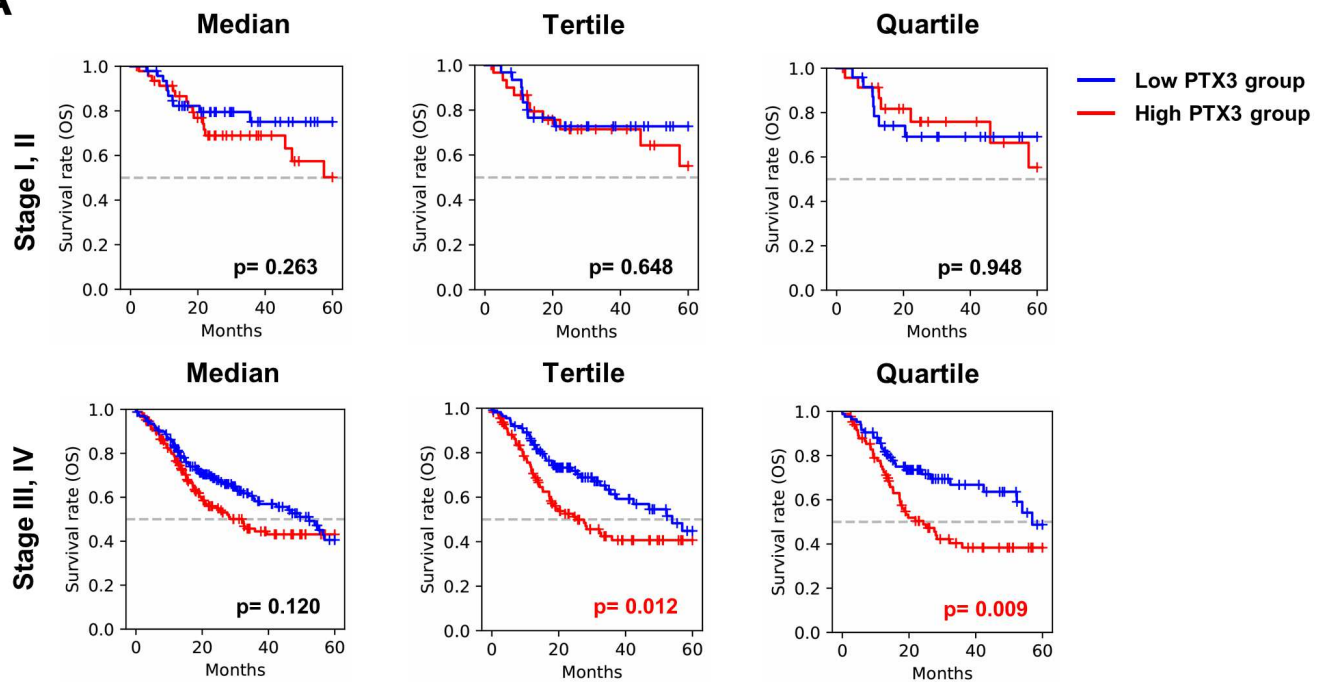
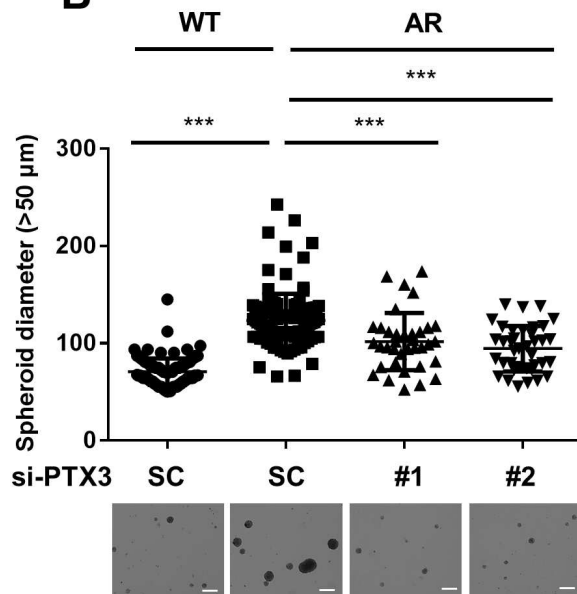


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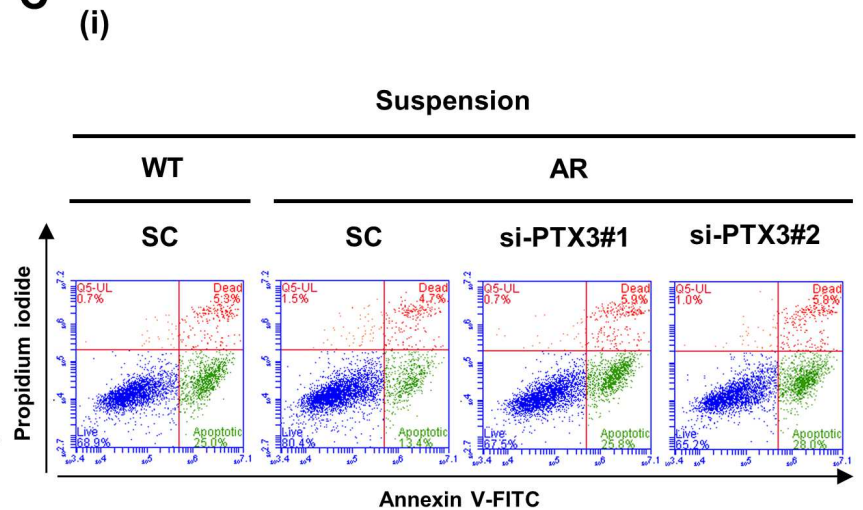
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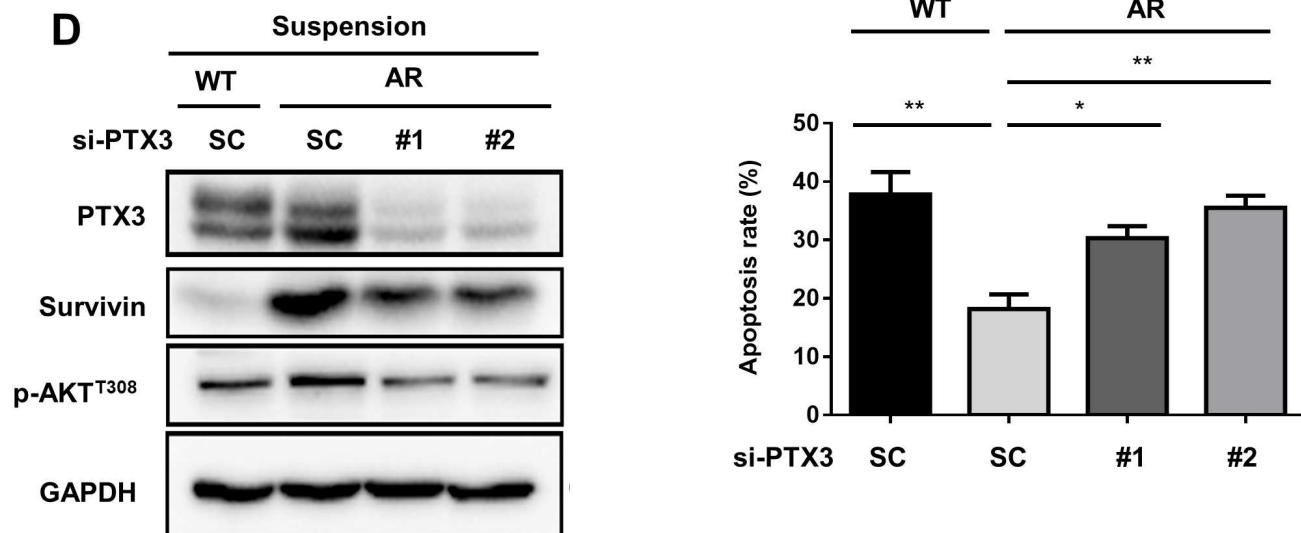


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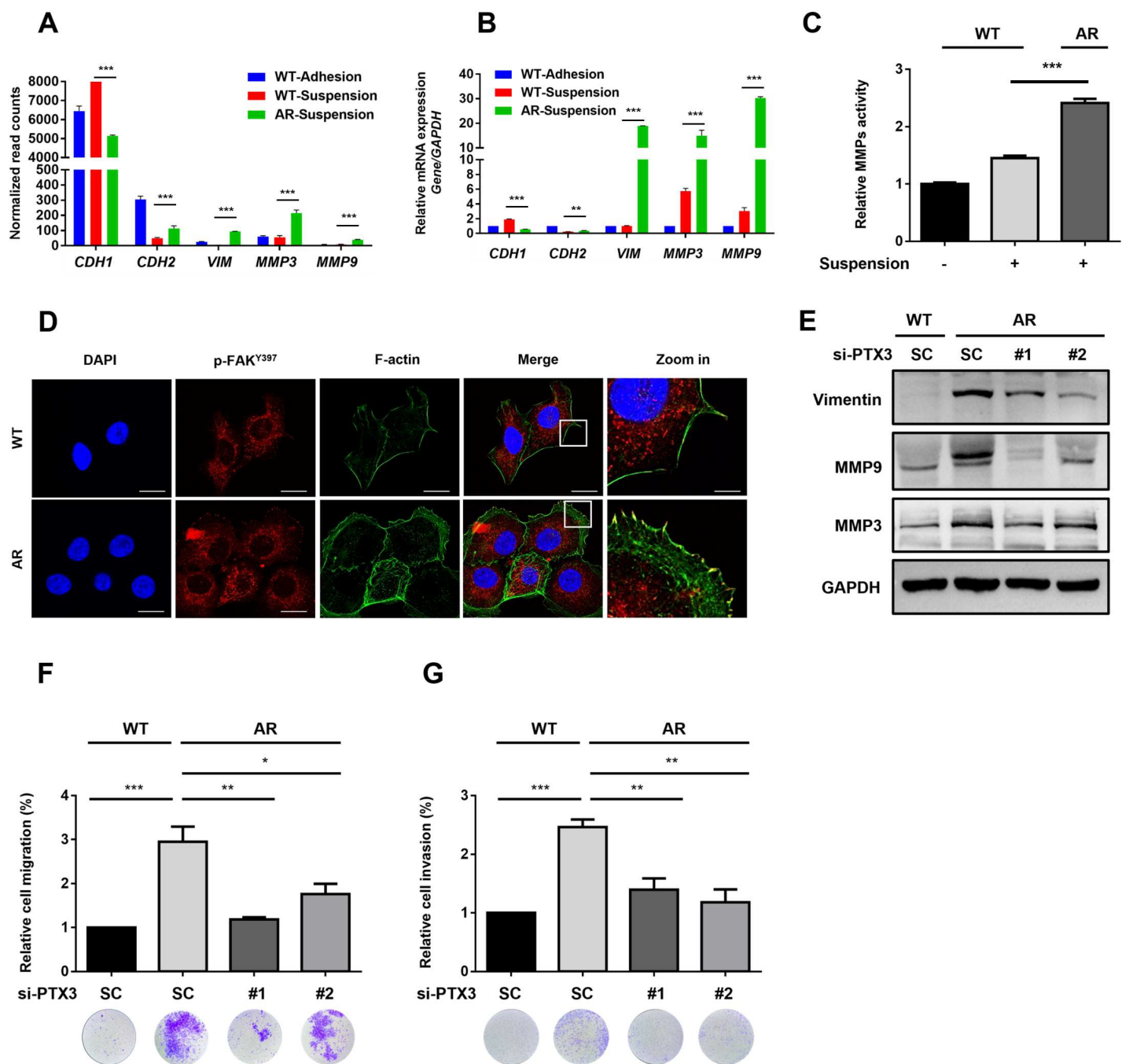


Figure 6

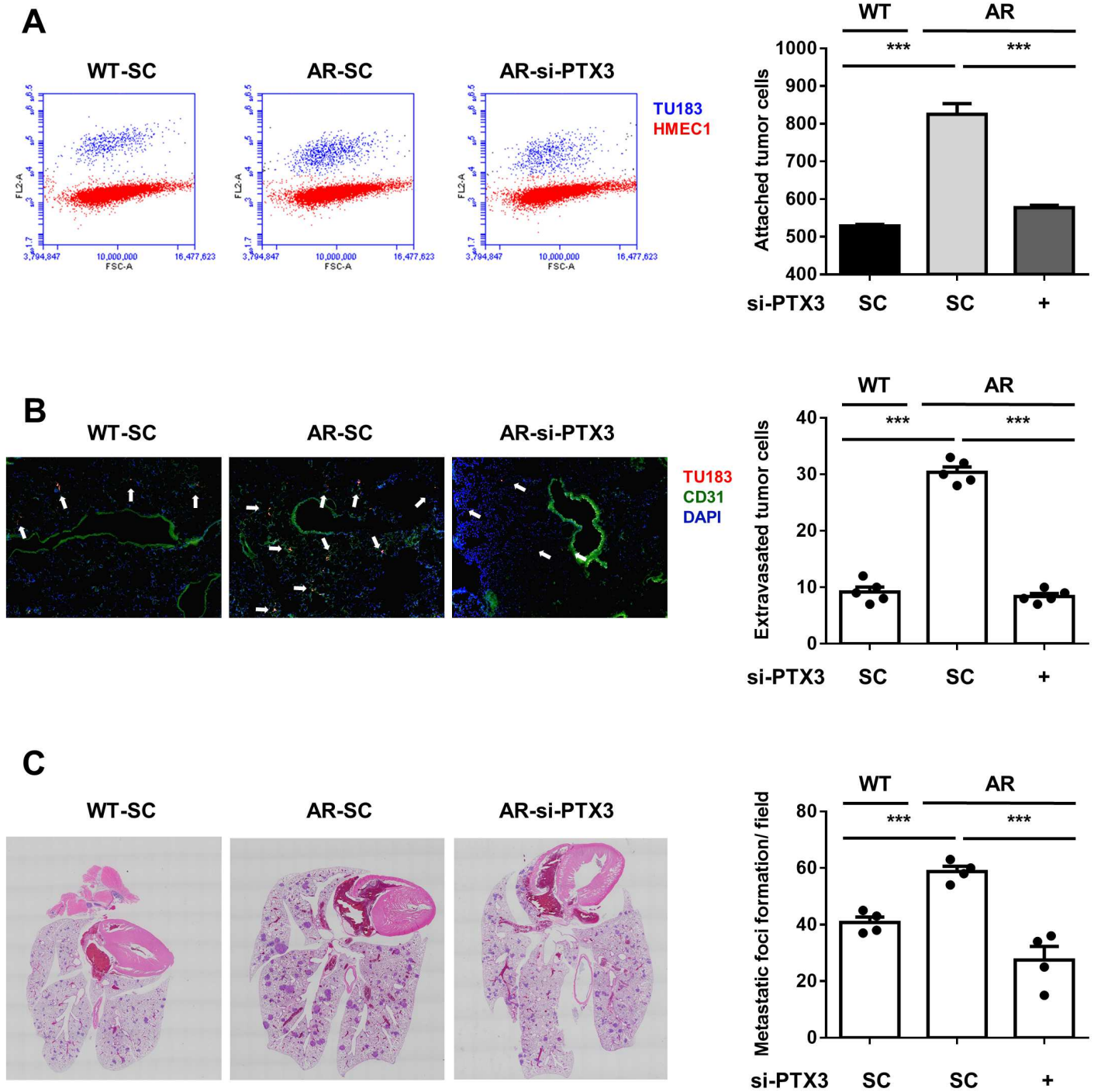


Figure 7

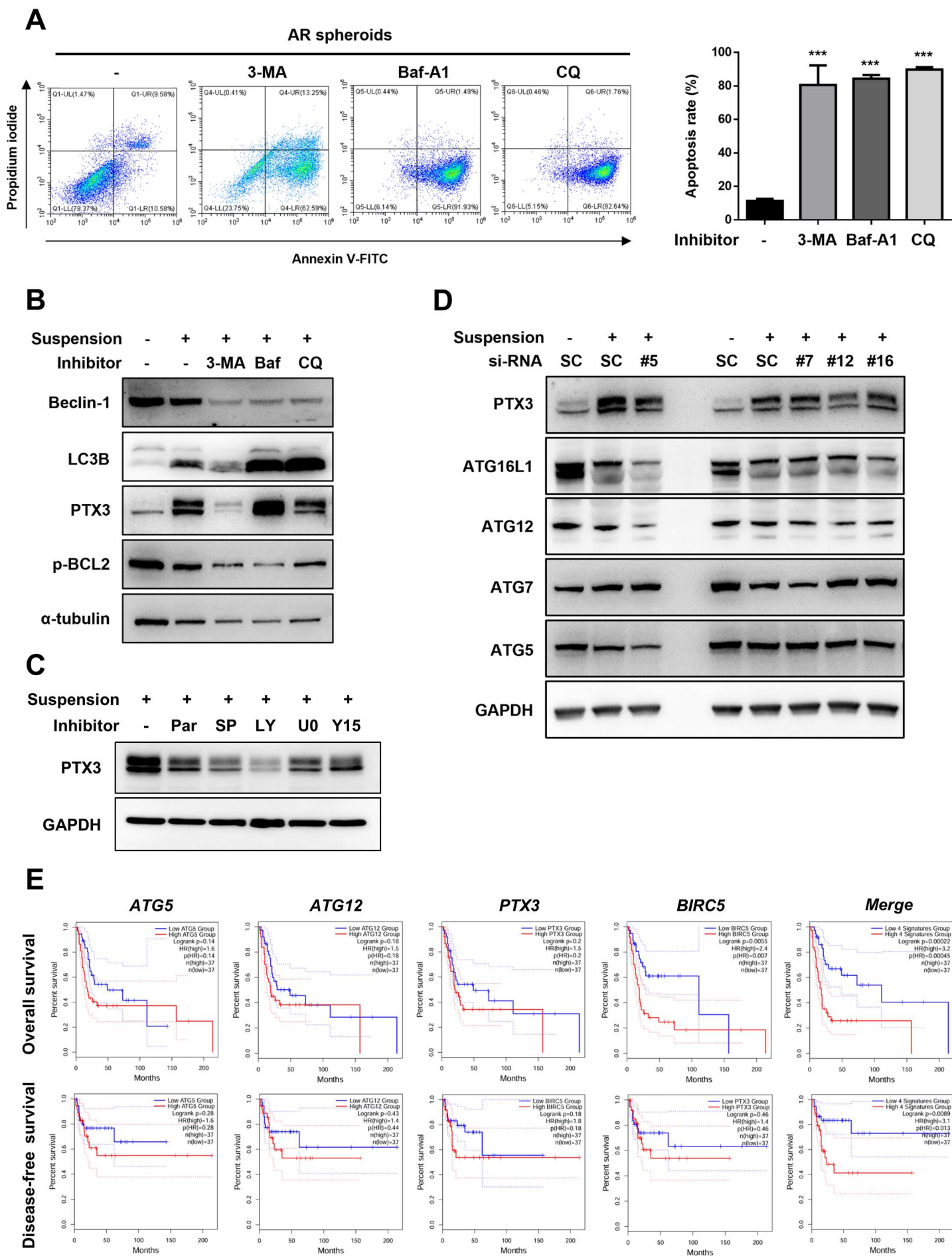
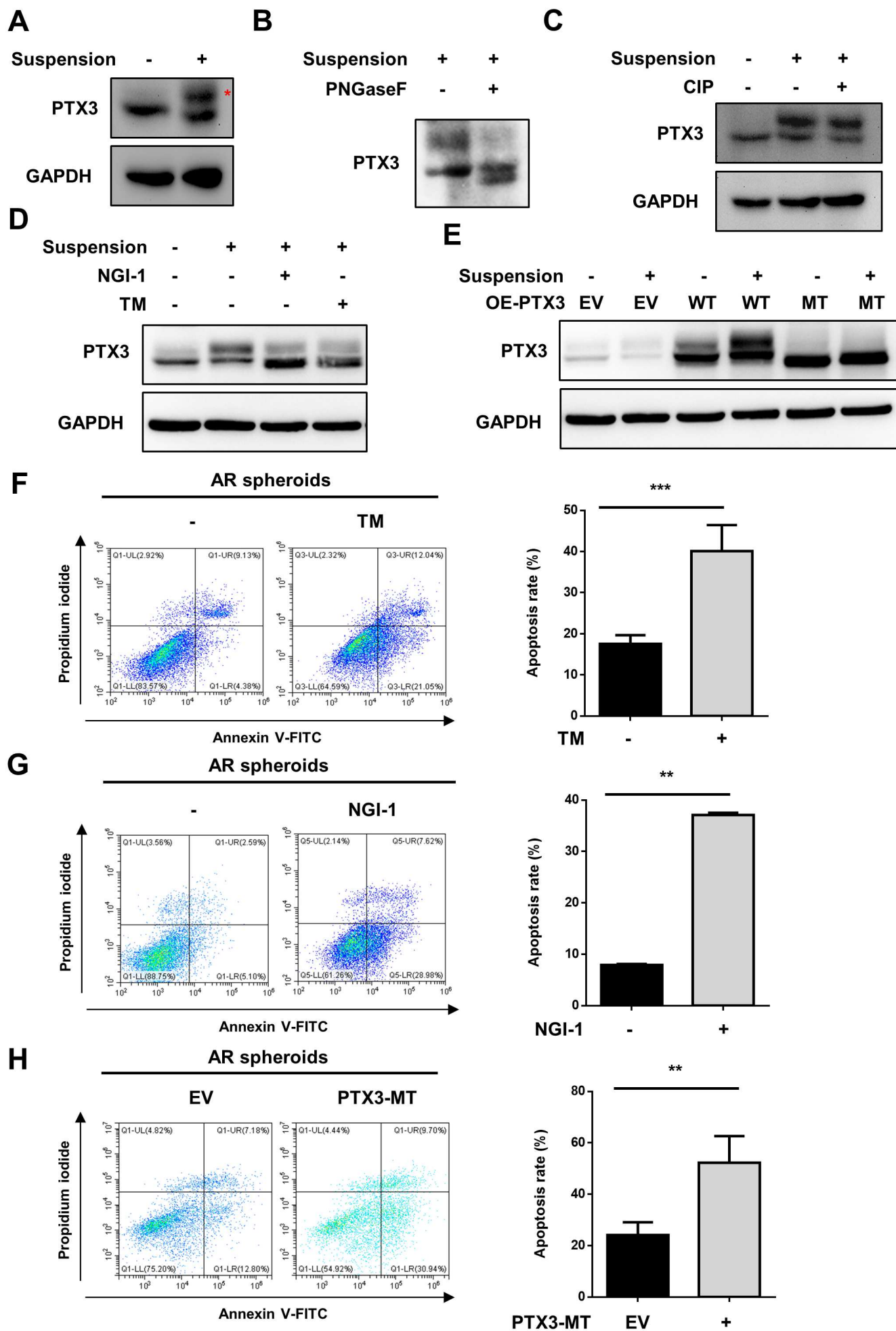


Figure 8



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