Supplemental Information

Novel ectopic expression of zona pellucida 3 glycoprotein in lung cancer promotes tumor growth

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- Supplementary Figures - S1 to S5
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- Supplementary Table
- Supplementary references

SUPPLEMENTARY FIGURES

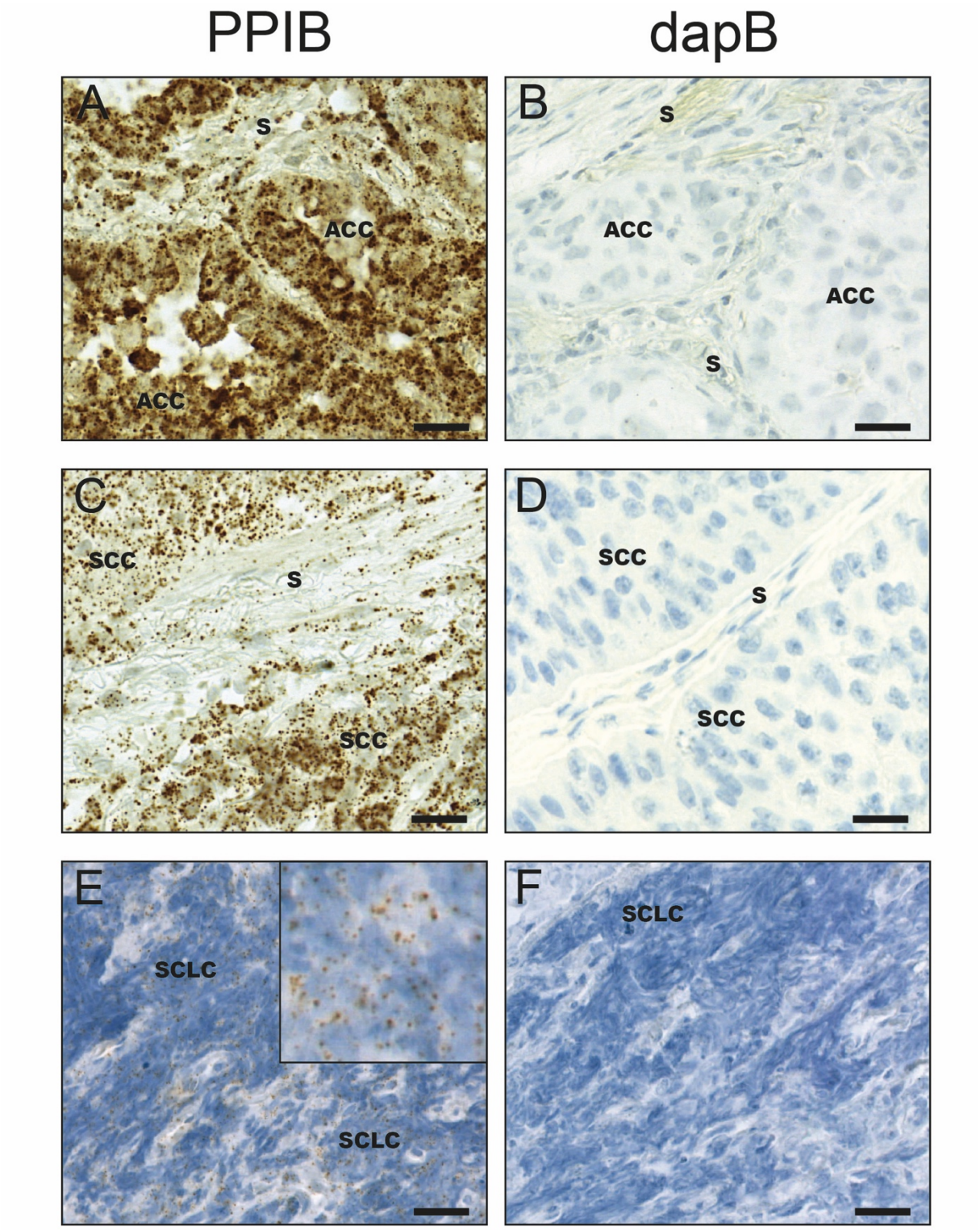

Fig S1

Fig. S1. *Positive and negative controls for RNAScope in situ hybridization in lung cancer tissues*

RNAScope *in situ* hybridization in lung adenocarcinoma (A, B), squamous cell lung carcinoma (C, D) and small cell lung carcinoma (E, F) using peptidylprolyl isomerase B (PPIB) positive control probe and (dapB) negative control probe, respectively. Each transcript is visible as a single brown dot in the *in situ* hybridization staining (bottom row). ACC, adenocarcinoma; SCC, squamous cell lung carcinoma; SCLC, small cell lung carcinoma; S, stroma. Original magnification, 40x; scale bar, 20 µm.

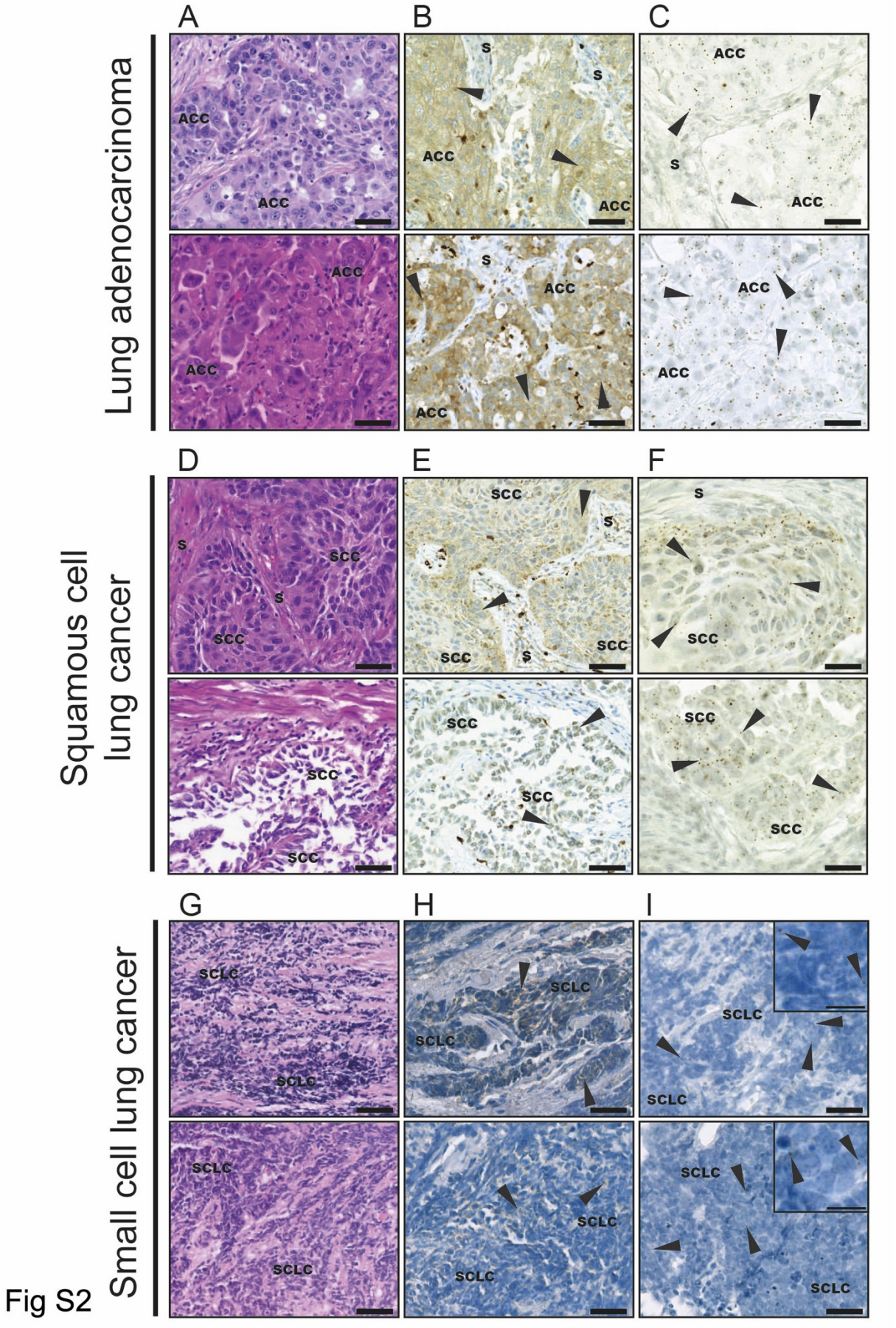
A
B
C
Lung adenocarcinoma
ACC
S
D
E
F
Squamous cell
lung cancer
SCC
G
H
I
Small cell lung cancer
SCLC

Fig S2

Fig. S2. *ZP3 expression at protein and mRNA level in lung cancer tissues of additional patients*
Histopathological analyses (A), immunohistochemical analyses of the ZP3 staining (B) and RNAScope in situ hybridization using ZP3 probe (C) in lung adenocarcinoma. Histopathological analyses (D), immunohistochemical analyses of the ZP3 staining (E) and RNAScope in situ hybridization using ZP3 probe (F) in squamous cell lung carcinoma. Histopathological analyses (G), immunohistochemical analyses of the ZP3 staining (H) and RNAScope in situ hybridization using ZP3 probe (I) in small cell lung carcinoma. Each transcript is visible as a single brown dot in the *in situ* hybridization staining. ACC, adenocarcinoma; SCC, squamous cell lung carcinoma; SCLC, small cell lung carcinoma; S, stroma. Original magnification, 40x; scale bar, 20 µm.

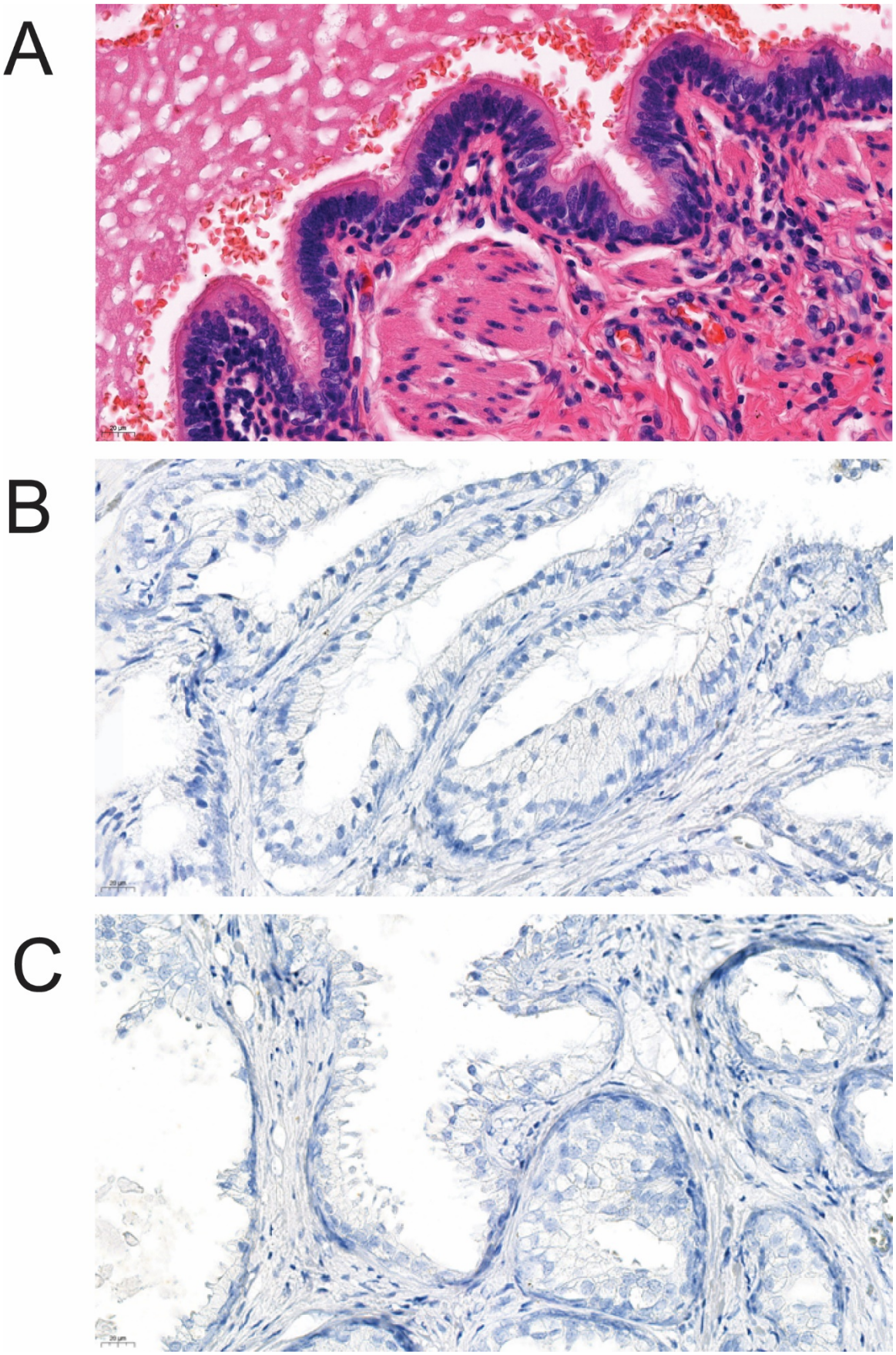
A
B
C

Fig. S3. *ZP3 expression at protein level in normal lung control in immunohistochemical studies.* Paraffin sections of normal human lung tissue: hematoxylin-eosin staining (A), incubated with ZP3 monoclonal antibody (B) or incubated with a non-immune immunoglobulin of the same isotype (IgG2A) in 1% BSA (C) at the same concentration as the primary ZP3 monoclonal antibodies. Then, sections were incubated with DAKO EnVision+ System – HRP labeled polymer and counterstained with hematoxylin. Scale bar, 20 μm.

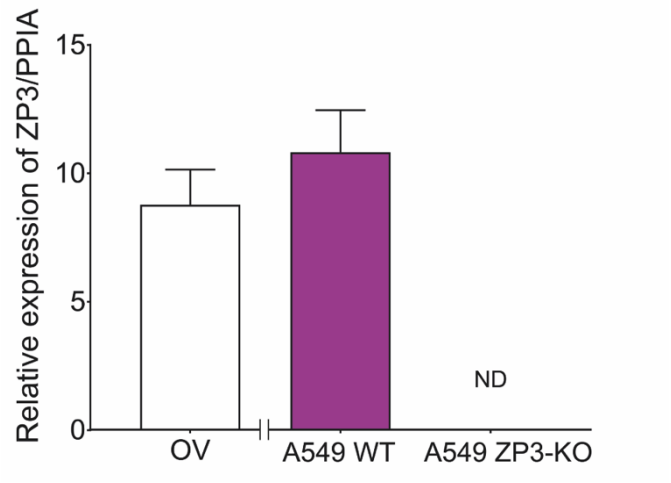

Fig. S4. *Relative expression of ZP3 in A549 ZP3-KO tumors*

qPCR analysis of the ZP3 expression levels in the A549 ZP3-KO vs. A549 WT tumors relative to peptidylprolyl isomerase A (PPIA) with ovary as a positive control tissue. Each bar represents the mean ± SD relative to PPIA. ND, not detected.

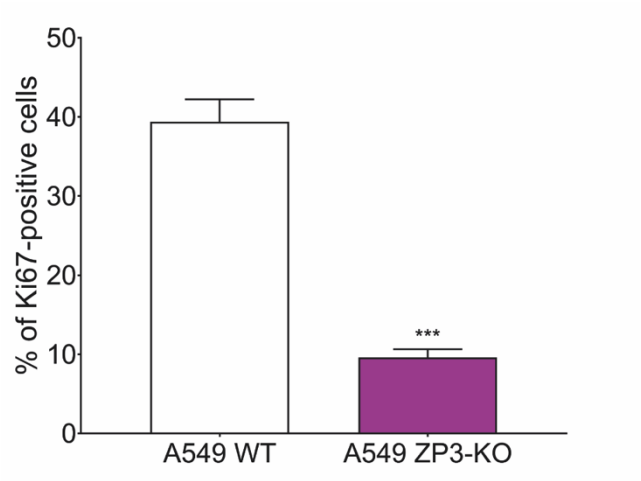

Fig. S5. *Densitometric analysis of the immunohistochemical stainings*

A densitometric analysis using ImageJ. Values calculated from the Ki-67 immunohistochemical staining of A549 WT and A549 ZP3-KO tumors. Each bar represents the mean ± SD of the cells recognized by the software as positively stained with the anti-Ki-67 antibody in the slide scans. Asterisks indicate significant differences between A549 ZP3-KO and A549 WT tumors (***, $P < .001$).

SUPPLEMENTAL MATERIALS AND METHODS

Cell viability assay

Cell viability was assessed using CellTiter 96® Aqueous One Solution Cell Proliferation Assay [MTS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (G3582, Promega Corporation, Madison, WI). Briefly, 0.02 x 10^6 cells of the A549 cell line (WT, D14 and D21 clone) were plated onto a 96-well plate in 28 replicates per clone. The top row of each plate was left empty as a blank control. The plate was incubated for 24 hat 37 °C in a humidified atmosphere in the presence of 5% CO_2. Next, 20 µl of combined MTS/PMS (PMS – phenazine methosulfate) solution was pipetted into each well of the 96-well plate containing 100 µl of cell culture medium. The plate was incubated at 37 °C in a humidified atmosphere in the presence of 5% CO_2 and gently shaken every 30 min. After 3.5 h, absorbance was measured at 490 nm using the EnSight Multimode Plate Reader (PerkinElmer, Waltham, MA).

Cell proliferation assay

Cell proliferation rate was assessed using BrdU Cell Proliferation Assay Kit (#6813, Cell Signaling Technology, Danvers, MA). Briefly, 0.02 x 10^6 cells of the A549 cell line (WT, D14, and D21 clones) were plated onto a 96-well plate in 28 replicates per clone. The top row of the plate was left empty as a blank control. The plate was incubated at 37 °C in a humidified atmosphere in the presence of 5% CO_2. Next, 10 µl of 10X BrdU solution was pipetted into each well of the 96-well plate containing 100 µl of cell culture medium. The plate was incubated for 24 h at 37°C in a humidified atmosphere in the presence of 5% CO_2. After the incubation, the cell culture medium was removed, 100 µl Fixing/Denaturing solution was added per well and

incubated for 30 min at room temperature (RT), then discarded. Next, 1X detection antibody solution was added (100 µl/well) and the plate was incubated for an hour at RT. Then, the solution was removed, and the plate was washed 3 times with 1X Wash Buffer. Next, 1X HRP-conjugated secondary antibody solution was added (100 µl/well), and the plate was incubated for 30 min at RT, then 3 times with 1X Wash Buffer. In the next step, 100 µl/well of TMB Solution was pipetted and the plate was incubated at RT. After 30 min, 100 µl/well of STOP solution was added and 450 nm absorbance was immediately measured using the EnSight Multimode Plate Reader (PerkinElmer, Waltham, MA).

Cell invasion assay

Cell migration rate was assessed using CultreCoat Medium BME Cell Invasion Assay kit (Catalog # 3482-096-K, R&D Systems, Inc., Minneapolis, MN). Before the assay, the A549 cells were cultured to 80% confluency, then starved for 18 h in Ham's F12K medium supplemented with 0.5% FBS. On the first day, a 96-well cell invasion chamber was brought to RT for an hour. The inserts were rehydrated by adding 25 µl/well of serum-free medium (at 37 °C) and incubated for an hour at 37 °C in a humidified atmosphere in the presence of 5% CO_2. In the meantime, A549 cells (WT, D14 and D21 clones) were harvested after starvation and diluted to a working concentration of 1 x 10^6 cells in a medium supplemented with 0.5% FBS. After rehydration, 3x 10^4 cells/well were seeded to each top chamber in 28 replicates per clone. The top row of the plate was left empty as a blank control. Next, 150 µl of cell culture medium without any chemoattractant was added to each bottom chamber. The chamber was assembled and incubated for 24 h at 37 °C in a humidified atmosphere in the presence of 5% CO_2. The next day, the contents of the top chamber were gently removed and each well was washed with 50 µl of 1X Wash Buffer. Then, the top chamber was transferred onto the black

receiver plate and 100 µl of Cell Disassociation Solution/Calcein AM mix was added to each well of the receiver plate. The plate was incubated for an hour at 37 °C in a humidified atmosphere in the presence of 5% CO_2. In the next step, the top chamber was gently tapped 10 times and removed from the receiver plate. Then, the plate was immediately read at 485 nm excitation and 520 nm emission wavelength using the EnSight Multimode Plate Reader (PerkinElmer, Waltham, MA).

RNA isolation

The TRIzol-based extraction method was used for total RNA isolation (Invitrogen, Carlsbad, CA). The quantity and quality of extracted RNA were determined by the A260/A280 absorbance measurement using NanoDrop (Thermo Scientific, Waltham, MA). Gel electrophoresis was performed to determine the integrity of isolated RNA.

Real-time quantitative PCR (qPCR)

For Real-time qPCR, the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) and the Bio-Rad CFX96 thermocycler (Bio-Rad Laboratories, Hercules, CA) were used. Reaction conditions were as follows: 10 min at 95 °C, 15 s at 95 °C, and 1 min at 60 °C up to 40 amplification cycles. PCR products were analyzed by melting curve analysis and agarose gel electrophoresis to ensure the amplification of a single product. Every reaction product was separated and verified by sequencing analysis. Expression levels of the investigated genes were normalized to the housekeeping genes peptidylprolyl isomerase A (PPIA), peptidylprolyl isomerase B (PPIB) and hypoxanthine phosphoribosyltransferase 1 (HPRT). The primer sequences were as follows:

human ZP3 F: TGGCAACAGCATGCAGGTA, R: CTGAGTGGCTGGGACTATGA;

human PPIA F: GTTCTTCGACATTGCCGTCG, R: TGTCTGCAAACAGCTCAAAGG;

human PPIB F: GGAGAGAAAGGATTTGGCTACA, R: GCTCACCGTAGATGCTCTTT.

RNA Scope in situ hybridization

Formalin-fixed paraffin-embedded tissue samples were handled according to the manufacturer's protocol using RNAscope 2.0 HD Assay (catalog number 310033, Advanced Cell Diagnostics [ACD], Hayward, CA). In brief, slides were deparaffinized in xylene (2 × 5 min), 100% EtOH (2 × 1 min), and air-dried for 5 min at room temperature. Each section was treated with hydrogen peroxide for 10 min at RT, then washed twice in distilled water. Slides were boiled in antigen retrieval buffer for 15 min and submerged in distilled water immediately thereafter. Next, the slides were washed in 100% EtOH and air-dried. For each section, barriers were drawn with the hydrophobic pen, and protease was applied for 30 min at 40 °C in HybEZ™ Oven (ACD). The slides were washed twice in distilled water. Probes for the targeted transcripts (human ZP3: ACD-442631) were applied as well as probes for positive (cyclophilin B–PPIB, a housekeeping gene, human ACD-313901) and negative controls (DapB – negative control probe targeting bacteria gene, ACD-310043). Then, the slides were incubated at 40 °C for 2 h in the oven and washed 2 × 2 min in the wash buffer. Thereafter, hybridization amplifiers (AMPs) were applied for 30 min (AMP 1, 3, 5) or 15 min (AMP 2, 4, 6) at 40 °C (AMP 1–4) or at room temperature (AMP 5 and 6) with double washing in between every step. After the last washing, equal volumes of BROWN-A and BROWN-B reagents were combined and applied to the sections for 10 min at RT. After double washing with distilled water, slides were counterstained in 50% Gill's hematoxylin (Vector Laboratories, Burlingame, CA, USA) for 2 min,

then washed in 0.02% ammonia water for 10 s and twice in distilled water. Dehydrated slides (2 × 2 min in 70% EtOH, 2 × 2 min in 100% EtOH, and 5 min in xylene) were mounted with Pertex (Histolab Products, Göteborg, Sweden).

Immunohistochemical staining

Monoclonal antibodies (isotype IgG1 kappa) specific to human ZP3 were produced with hybridoma techniques using CRL-2569 hybridoma (ATCC, Manassas, VA) as described previously [1]. Formalin-fixed paraffin-embedded (FFPE) samples were deparaffinized and hydrated. Slides were boiled for 15 min in antigen retrieval buffer (10 mM citric acid buffer with 0.05% Tween20, pH = 6). Then, the sections were incubated in a humidified chamber for 1 h at RT with 3% BSA for reducing nonspecific background staining. Next, slides were incubated overnight in a humidified chamber at 4 °C with the primary antibody anti-ZP3 (0.5 µg/mL) or anti-Ki67 (1:100) (GA62661-2, DAKO, Agilent, Santa Clara, CA). Endogenous peroxidase activity was blocked by incubating slides in 0.5% H_2O_2 in PBS at room temperature for 20 min. DAKO polymer (DAKO EnVision + System – HRP labeled polymer) was applied to each section and incubated in a humidified chamber for 30 min at room temperature. DAB + Chromogen (DAKO) was applied for 5 min. Slides were washed in dH2O, counterstained in Mayer's hematoxylin (Sigma-Aldrich, Saint Louis, MO), dehydrated and mounted with Pertex (Histolab Products). As a control for the antibodies, tissues were incubated with 3% BSA and DAKO polymer to differentiate unspecific from specific staining.

Immunocytochemical staining

A549 WT, D14 and D21 cells were cultured on Nunc™ Lab-Tek™ Chamber Slide System (177380, Thermo Scientific, Waltham, MA) and were washed twice with PBS and fixed in ice-cold

acetone for 10 min on ice. The cells were washed 2 x 3 min with PBS, then permeabilized with 0,1% Triton-X and washed 3 x 3 min in PBST. To reduce autofluorescence, the cells were treated with 100 mM NH_4Cl for 10 min and washed 3 x 3 min in PBST. In the next step, cells were incubated with 3% BSA in PBST for 30 min at RT to block unspecific binding. Thereafter, cells were incubated for 1 h with primary monoclonal anti-ZP3 antibody (the same antibody as used in immunohistochemistry) diluted in a blocking solution. After washing 3 x 3 min with PBST, cells were incubated with Alexa Fluor 488 goat anti-mouse IgG (A11029; Thermo Fisher, Waltham, MA; dilution 1:500) for 45 min at RT in the dark and washed again. DAPI dye was used as a counterstain to detect cell nuclei. As a control for the antibodies, the cells were incubated with either 3% BSA or Alexa Fluor-488 goat anti-mouse IgG as a primary antibody to differentiate unspecific from specific staining.

Total RNA sequencing

Total RNA sequencing was performed to identify differentially expressed genes (DEGs) and the molecular pathways they affect. The preparation of RNA library and transcriptome sequencing was conducted by Novogene Co., LTD (Beijing, China). Libraries were sequenced using a NovaSeq 6000 PE150 instrument (Illumina, Inc., San Diego, CA). Data were filtered and further mapped to the reference genome using HISAT2 software [2]. The differential expression analysis was carried out using DESeq2 and EdgeR algorithms based on FPKM+20 counts to eliminate noise signals from low-expressing genes. Genes with adjusted p-value < 0.05 and $|\log2(\text{FoldChange})| > 0$ were considered differentially expressed.

Optical density analysis

A densitometric analysis using ImageJ [3]. Values were calculated from the Ki-67 immunohistochemical staining of A549 WT and A549 ZP3-KO tumors (n = 10 each). The area

of the cells recognized by the software as positively stained with the anti-Ki-67 antibody was compared to the total area of the tissue in the slide scans. The final value is presented as a percentage of area positively stained with the anti-Ki-67 antibody.

Table S1

Quality control assessment of the total RNA sequencing data.

Sample name	Total number of sequenced reads	Total number of uniquely mapped reads (GRCh38)	RNA integrity number (RIN)	Ratio of all reads aligned to rRNA regions to total uniquely mapped reads (rRNA rate)	Ratio of exon-mapped reads to total uniquely mapped reads (Expression Profile Efficiency)	Total number of detected genes with reads ≥1
WT_1	78,823,508	74,062,086	9,8	97.45%	93.96%	23,066
WT_2	76,866,624	72,172,486	9,7	97.37%	93.89%	25,026
WT_3	77,691,662	72,810,674	9,7	97.42%	93.72%	23,787
KO_1	86,161,654	80,494,862	9,6	97.31%	93.42%	25,196
KO_2	95,013,936	88,588,822	9,9	97.14%	93.24%	26,080
KO_3	78,002,918	72,673,664	9,7	97.92%	93.17%	23,573

Supplemental references

1 Pulawska K, Ponikwicka-Tyszko D, Lebiedzinska W, Guo P, Bernaczyk P, Pilaszewicz-Puza A *et al*. Novel expression of zona pellucida 3 protein in normal testis; potential functional implications. *Mol Cell Endocrinol* 2022; 539: 111502.

2 Zhang Y, Park C, Bennett C, Thornton M, Kim D. Rapid and accurate alignment of nucleotide conversion sequencing reads with HISAT-3N. *Genome Res* 2021; 31: 1290-1295.

3 Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012; 9: 671-675.