




















## RESEARCH ARTICLE

## Molecular Cancer Biology

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

Kamila Pulawska-Moon<sup>1</sup>  | Donata Ponikwicka-Tyszko<sup>1,2</sup>  |  
 Weronika Lebiedzinska<sup>3</sup>  | Agata Pilaszewicz-Puza<sup>4</sup>  | Piotr Bernaczyk<sup>4</sup>  |  
 Mariusz Koda<sup>5</sup>  | Oana Lupu<sup>3</sup>  | Gabriela Milewska<sup>3</sup>  | Chen-Che Jeff Huang<sup>6</sup>  |  
 Huifei Zheng<sup>6</sup>  | Phillip Schiele<sup>7</sup>  | Il-Kang Na<sup>7,8,9</sup>  | Marco Frentsch<sup>7</sup>  |  
 Xiangdong Li<sup>3,10</sup>  | Jorma Toppari<sup>1,11</sup>  | Sławomir Wolczynski<sup>3</sup>  |  
 Herjan J. T. Coelingh Bennink<sup>12</sup>  | Ilpo Huhtaniemi<sup>1,13</sup>  | Nafis A. Rahman<sup>1,3</sup> 

<sup>1</sup>Institute of Biomedicine, University of Turku, Turku, Finland

<sup>2</sup>Department of Biology and Pathology of Human Reproduction, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland

<sup>3</sup>Department of Reproduction and Gynecological Endocrinology, Medical University of Białystok, Białystok, Poland

<sup>4</sup>Department of Pathomorphology, Medical University of Białystok, Białystok, Poland

<sup>5</sup>Department of General Pathomorphology, Medical University of Białystok, Białystok, Poland

<sup>6</sup>Department of Anatomy, Physiology and Pharmacology, Auburn University College of Veterinary Medicine, Auburn, Alabama, USA

<sup>7</sup>BIH Center for Regenerative Therapies (BCRT), Berlin Institute of Health at Charité—Universitätsmedizin Berlin, Berlin, Germany

<sup>8</sup>Department of Hematology, Oncology and Tumor Immunology, and ECRC Experimental and Clinical Research Center, both Charité—Universitätsmedizin Berlin, Corporate members of Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany

<sup>9</sup>German Cancer Consortium (DKTK), Berlin, Germany

<sup>10</sup>State Key Laboratory of Agro-Biotechnology, China Agricultural University, Beijing, China

<sup>11</sup>Department of Pediatrics, Turku University Hospital, Turku, Finland

<sup>12</sup>Pantarhei Oncology/Bioscience, Zeist, Netherlands

<sup>13</sup>Institute of Reproductive and Developmental Biology, Imperial College London, London, UK

## Correspondence

Nafis A. Rahman, Institute of Biomedicine,  
University of Turku, 20520 Turku, Finland.  
Email: [nafis.rahman@utu.fi](mailto:nafis.rahman@utu.fi)

## Funding information

Medical University of Białystok, Grant/Award  
Number: SUB/1/DN/20/005/1104; Ella and  
Georg Ehrnrooth Foundation; Turun  
Yliopistollisen Keskussairaalan Koulutus- ja  
Tutkimussäätiö; Pantarhei Bioscience/  
Oncology; Magnus Ehrnrooth Foundation

## Abstract

Zona pellucida 3 (ZP3) expression is classically found in the ZP-layer of the oocytes, lately shown in ovarian and prostate cancer. A successful ZP3 ovarian cancer immunotherapy in transgenic mice suggested its use as an attractive therapeutic target. The biological role of ZP3 in cancer growth and progression is still unknown. We found that ~88% of the analyzed adenocarcinoma, squamous and small cell lung carcinomas to express ZP3. Knockout of ZP3 in a ZP3-expressing lung adenocarcinoma cell line, significantly decreased cell viability, proliferation, and migration rates in vitro. Zona pellucida 3 knock out (ZP3-KO) cell tumors inoculated in vivo in immunodeficient non-obese diabetic, severe combined immunodeficient mice showed significant inhibition of tumor growth and mitigation of the malignant phenotype. RNA sequencing revealed the deregulation of cell migration/adhesion signaling pathways in ZP3-KO cells.

This is an open access article under the terms of the [Creative Commons Attribution](https://creativecommons.org/licenses/by/4.0/) License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2024 The Author(s). *International Journal of Cancer* published by John Wiley & Sons Ltd on behalf of UICC.

This novel functional relevance of ZP3 in lung cancer emphasized the suitability of ZP3 as a target in cancer immunotherapy and as a potential cancer biomarker.

#### KEYWORDS

cancer growth, lung cancer, zona pellucida 3

#### What's New?

The protein ZP3 is frequently expressed in lung cancers, but its function is not well understood. However, it is an attractive therapeutic target, and an ovarian cancer immunotherapy against ZP3 has been successful in mice. Here, the authors show that knocking out ZP3 in a lung adenocarcinoma cell line decreased cell viability and migration rates. In mice, tumors lacking ZP3 did not grow well. RNA sequencing revealed that without ZP3, cell migration and adhesion pathways were deregulated.

## 1 | INTRODUCTION

Lung cancers are one of the leading causes of cancer-related deaths worldwide.<sup>1</sup> There are two major types of lung cancer: small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC).<sup>2</sup> NSCLCs have a higher prevalence and occur as one of three histopathological subtypes: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.<sup>2</sup> Recently, an increased incidence of lung adenocarcinoma in the general population caused this to become the most prevalent type of NSCLC.<sup>3</sup> The current therapies for lung cancer encompass surgery, radiotherapy, and chemotherapy.<sup>4</sup> It is becoming more common to perform genotyping or RNA sequencing of cancer due to genomic alterations that cause aberrant activation of proliferation pathways and evasion of apoptotic signaling.<sup>5</sup> This in-depth insight into the cancer's genetic makeup may help develop a personalized treatment plan for the patient, such as immunotherapy approach.

The extracellular layer of the zona pellucida (ZP) surrounds mammalian oocytes and in human is composed of four glycoproteins: ZP1, ZP2, ZP3, and ZP4.<sup>6</sup> ZP3 plays an important role in oogenesis, prevention of polyspermy and in the early embryo developmental stages.<sup>6</sup> During the initiation of folliculogenesis, a small portion of primary follicles is recruited.<sup>7</sup> ZP proteins start being transcribed in growing oocytes up to the first reductive meiotic division and afterwards, their transcription ceases.<sup>7</sup> The transcription of the ZP proteins is coordinated by folliculogenesis specific basic helix-loop-helix transcription factor (FIGLA) transcription factor (Folliculogenesis Specific Basic Helix-Loop-Helix Transcription Factor).<sup>8</sup> ZP3 colocalize with the endoplasmic reticulum and Golgi apparatus, where they undergo post-translational modifications, such as N-glycosylation.<sup>9</sup> ZP3 glycoprotein is essential for oocyte fertilization and for the activation of the acrosomal reaction in humans.<sup>10</sup> It has been shown that the ZP3-knockout female mice are infertile and in humans, the ZP3 gene mutations cause empty follicle syndrome.<sup>11,12</sup> Considering the critical role of ZP3 in the fertilization process, both native and recombinant ZP3 immunization has been successfully used as a contraception strategy in wild-life animal populations.<sup>13</sup> However, these wild-life ZP3 immunizations resulted in a loss of ovarian function due to

iatrogenic autoimmune oophoritis (IAO) mediated by ZP3-specific auto-reactive T cells, which prevented further human trials.<sup>13,14</sup> A specific selection of ZP3 epitopes was found to induce infertility in animal models without ovarian deleterious effects.<sup>15,16</sup> Recently, we have also demonstrated ZP3 expression in human and murine testis, but not in any other healthy tissues.<sup>17</sup>

An abundant expression of ZP3 has been found in ovarian epithelial and granulosa cell cancers, their metastases, and ovarian cancer cell lines, as well as in prostate cancer.<sup>18–20</sup> ZP3 expression in lung and colorectal cancers has been reported in patent applications.<sup>21–23</sup> There is also a recent report on ZP3 expression in hepatocellular carcinoma cells and its potential association with cirrhosis and hepatitis B infection in hepatocellular carcinoma patients.<sup>24</sup> A successful proof-of-concept immunization strategy against ZP3 in a malignant ovarian cancer in a transgenic mouse model established the concept of ZP3 as an attractive tumor-specific antigen target for cancer immunotherapy treatment.<sup>18</sup> Until now, the potential functional role of ZP3 in cancer, as well as in tumor progression is not known—pivotal information for any future clinical ZP3 immunization development.

In this study, we analyzed in-depth the functional role of ZP3 in lung cancer by generating an in vitro knockout model using the CRISPR/Cas9 technology in a lung adenocarcinoma A549 cell line endogenously expressing ZP3. We also checked the in vivo tumorigenic property of WT and knockout (ZP3-KO) lung adenocarcinoma A549 cells in the NOD SCID (non-obese diabetic, severe combined immunodeficient) mouse model and explored the signaling pathways involved.

## 2 | MATERIALS AND METHODS

Please also refer to Pulawska et al. 2021<sup>17</sup> as well as the Supplemental Materials and Methods (Data S1), where methods for cell viability assay, cell proliferation assay, cell invasion assay, RNA isolation, quantitative PCR, RNAScope in situ hybridization, immunohistochemical staining, immunocytochemical staining, and total RNA sequencing have been described.

## 2.1 | Human tissue samples

Archival formalin-fixed paraffin-embedded human tissue block samples or frozen tissues were obtained from the Department of Pathomorphology and/or University Biobank, Medical University of Białystok, Poland. The examined samples contained: lung adenocarcinoma  $n = 14$  (10 males and 4 females); squamous cell lung carcinoma  $n = 14$  (8 males and 6 females); small cell lung carcinoma  $n = 17$  (12 males and 5 females). All archival materials were  $\leq 5$  years old at the time of the analysis. The samples were taken for diagnostic (small cell lung carcinoma) and/or treatment (i.e., surgery, for almost all other types) purposes from patients ages 32–68 (median 43). Histopathological analysis was done by a pathologist for all the samples. The human ovarian samples were from the Medical University of Białystok Biobank. The normal ovaries ( $n = 5$ ) were removed during the surgery from patients with BRCA1 or BRCA2 gene mutations undergoing a prophylactic salpingo-oophorectomy (age between 24 and 36, BMI normal, inclusion and exclusion criteria: no urogenital cancer, no endometriosis, no polycystic ovarian syndrome). A prophylactic salpingo-oophorectomy has been shown to reduce the risk of ovarian cancer by more than 96% and also reduces the overall cancer-specific mortality in patients with BRCA1 or BRCA2 gene mutations.<sup>25–28</sup>

## 2.2 | Xenograft tissue samples

NOD SCID mice ( $n = 10$ , defined after power analysis) were subcutaneously inoculated with A549 cells on the upper back. Wild-type (WT) lung adenocarcinoma A549 cells were placed on the left side. Clone D14 of the CRISPR/Cas9 ZP3 knockout in A549 cells (ZP3-KO) was placed on the right side. For detailed information on A549 WT (WT) and D14 clone (ZP3-KO), please see Supplemental Materials (Data S1).

## 2.3 | Cell culture and CRISPR/Cas9 knockout of lung adenocarcinoma cell line

The human lung adenocarcinoma cell lines (ATCC, Manassas, VA) were cultured in the cell culture medium (GIBCO, Paisley, UK) as recommended by ATCC. NCI-H2030 (RRID:CVCL\_1517)<sup>29</sup> and NCI-H1975 (RRID:CVCL\_1511),<sup>30</sup> were cultured in RPMI1640 medium, A-549 (RRID:CVCL\_0023)<sup>31</sup> was cultured in Ham's F12K medium and Calu-3 (RRID:CVCL\_0609)<sup>32</sup> was cultured in EMEM. All cell culture media were supplemented with 10% fetal bovine serum (FBS; Biochrom, Berlin, Germany) and 100 units/mL penicillin, and 100  $\mu\text{g/mL}$  streptomycin (P/S solution; Sigma-Aldrich, Saint Louis, MO). All cell lines were cultured at 37°C in a humidified atmosphere in the presence of 5% CO<sub>2</sub>. All human cell lines have been authenticated using STR (short tandem repeats) profiling within the last 3 years. All experiments were performed with mycoplasma-free cells. Three independent cell platings were

performed in triplicates for each RNA isolation and immunocytochemistry study. The A549 cell line was chosen as a model in this study, as we found relatively high endogenous ZP3 expression in them and better performance in CRISPR/Cas9 transfection, compared to the other cell lines checked in this study.

The two ZP3 knockout single-cell clones of the A549 cell line (D14 and D21) were obtained using CRISPR/Cas9 technology (acquired through a guaranteed service from Synthego Inc., Menlo Park, CA). The A549 cell transfection was approached with the multiple guide RNA targeting exon 5 incorporated into ribonucleoprotein complexes. No selection agents were applied after the transfection. Both clones were genotyped by the company, where clone D14 had a 14 nt deletion in the ZP3 genomic sequence, whereas clone D21 had a 1 nt deletion. The culture conditions for the clones were identical to the wild-type (WT) A549 cells.

The sequencing was performed by Novogene Co., LTD (Beijing, China). Libraries were sequenced using a NovaSeq 6000 PE150 instrument (Illumina, Inc., San Diego, CA). The sequencing coverage and quality statistics for each sample are summarized in Table S1.

## 2.4 | Statistical analysis

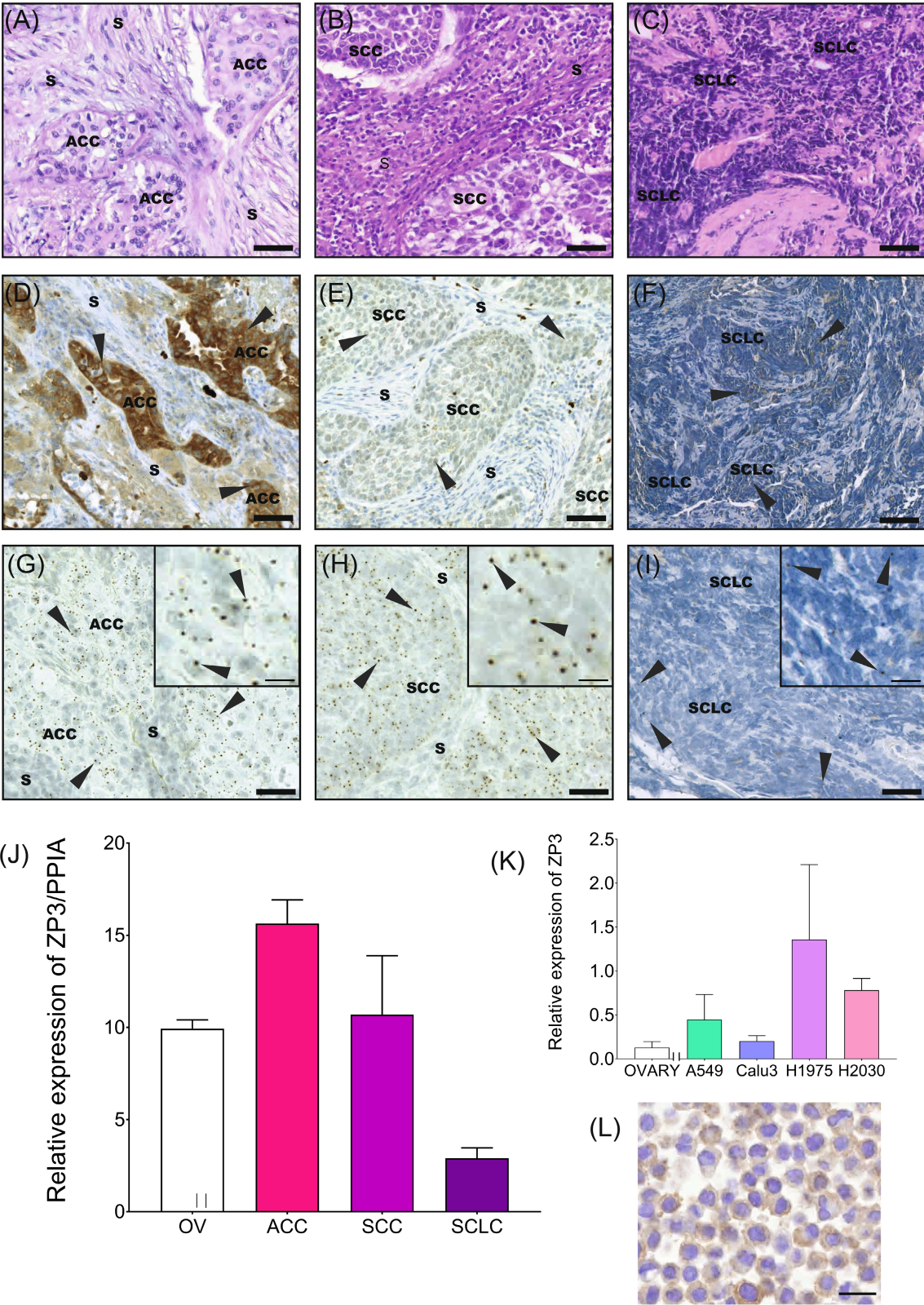
Numerical data are presented as mean  $\pm$  SD. To analyze statistical significance, one-way ANOVA or Kruskal–Wallis test with the post hoc Bonferroni's multiple comparison post hoc test with 95% confidence interval was applied for functional assays, whereas, *t*-test or Kolmogorov–Smirnov test was applied for the relative expression analyses (GraphPad PRISM v. 7.05. GraphPad Software Inc., San Diego, CA, USA). Results were considered to be statistically significant at  $p < .05$  level.

## 3 | RESULTS

### 3.1 | ZP3 expression in lung carcinomas and human lung adenocarcinoma-derived cell lines

ZP3 expression was found in  $\sim 88\%$  of the different types of lung cancer cases [adenocarcinoma,  $n = 12/14$  (85.8%), squamous cell,  $n = 12/14$  (85.8%), and small cell lung carcinoma,  $n = 15/17$  (88.2%)], at mRNA and protein levels (Figure 1A–I). Densitometric analysis of the immunohistochemical staining revealed the highest ZP3 expression in adenocarcinoma and the lowest in small cell carcinoma (Figure 1D–F, respectively). Similarly, RNAscope in situ hybridization localized ZP3 mRNA transcripts in lung adenocarcinoma, squamous cell lung carcinoma and small cell lung carcinoma (Figure 1G–I, respectively). The positive and negative control stainings for IHC (immunohistochemistry) and ISH (in situ hybridization) are included in Figure S1. Additional ZP3 immunohistochemistry staining and in situ hybridization of lung carcinoma samples and healthy lung are shown in Figures S2 and S3, respectively. A qPCR analysis confirmed the ZP3 expression in lung adenocarcinoma,





**FIGURE 1** Legend on next page.



squamous cell lung carcinoma and small cell lung carcinoma (Figure 1J). ZP3 expression was also found in all of the analyzed human lung adenocarcinoma-derived cell lines, such as A549, Calu3, H1975 and H2030. A human ovary was used as a positive control for the ZP3 expression (Figure 1K). Additionally, immunohistochemical staining showed an abundant ZP3 signal in the cytoplasm of A549 cells (Figure 1L).

### 3.2 | Knockout of the ZP3 gene impaired A549 lung cancer cells' viability, proliferation, and invasion rates

To investigate the functional role of ZP3 in lung cancer, the ZP3 gene was knocked out in A549 cells by the CRISPR/Cas9 method. Both qPCR and immunocytochemical examinations confirmed that ZP3 was successfully knocked out in A549 WT cells (Figure 2A). Both A549 D14 and A549 D21 clones showed no ZP3 expression at protein (Figure 2B,C) and mRNA level (Figure 2D). A549 D14 ZP3-KO, as well as, A549 D21 ZP3-KO clones showed inhibition of cell viability (Figure 2E) and cell proliferation (Figure 2F), compared to A549 WT cells. Cell invasion was significantly decreased only in the A549 D14 ZP3-KO clone cells, but not in the A549 D21 clone. (Figure 2G). As the D14 clone was more stable and reliable, further investigations were carried out on the A549 D14 clone, referred to as A549 ZP3-KO.

### 3.3 | Altered ZP3 expression affected/deregulated angiogenesis, cell adhesion, and cell migration pathways in lung cancer cells

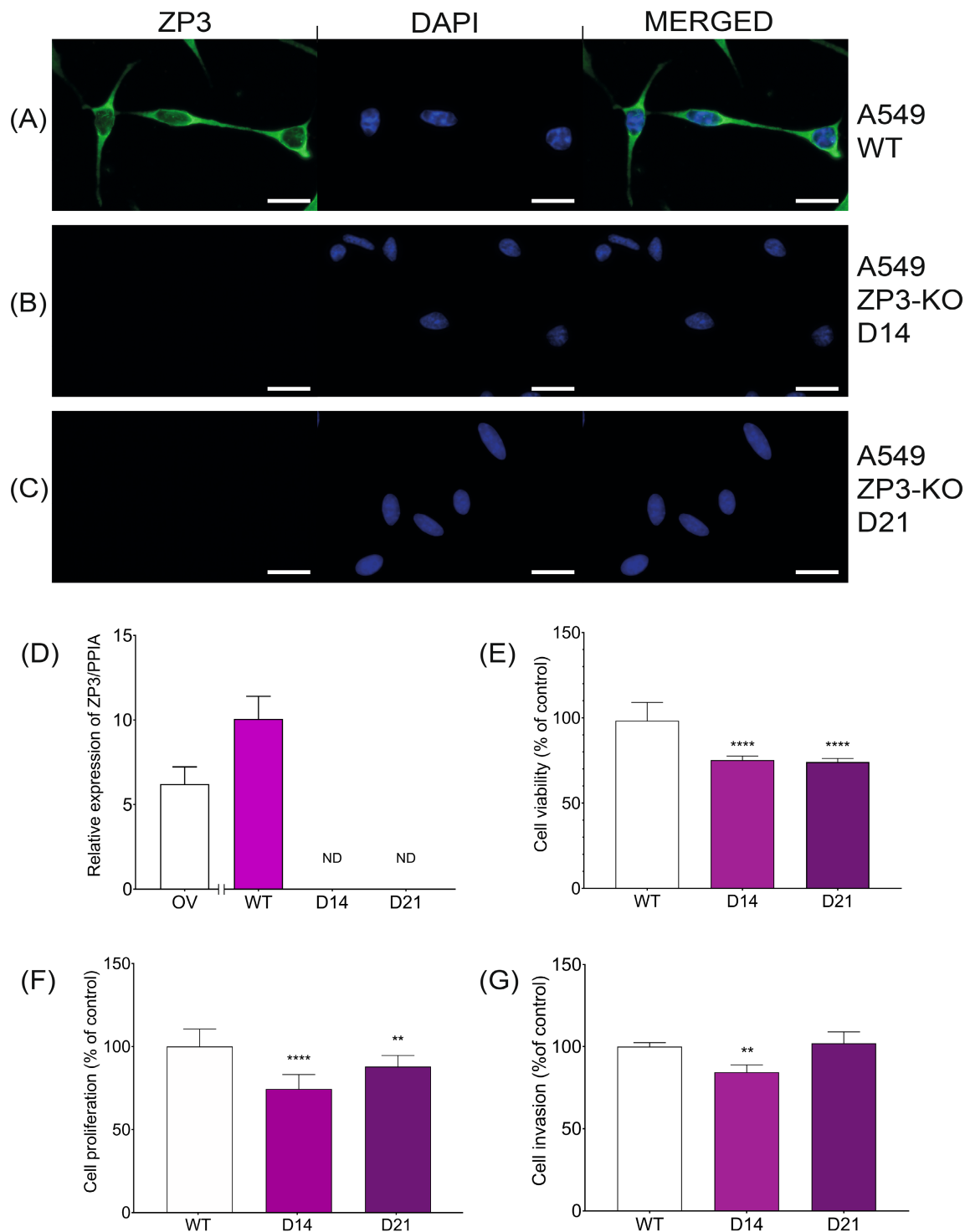
To further select and analyze signaling pathways that may potentially be regulated by ZP3, total RNA next-generation sequencing (NGS) was performed in the A549 ZP3-KO ( $n = 3$ ) and A549 WT ( $n = 3$ ) cells from three different passage numbers. Sequencing result analysis revealed a variety of differentially expressed genes (DEGs) in A549 ZP3-KO cells, compared to A549 WT cells. The total number of identified genes was 58,735 (Figure 3A). Concordance of two algorithms found 555 differentially expressed genes (Figure 3A). DESeq2 analysis identified 60 unique DEGs, whereas EdgeR analysis found 91 unique DEGs in ZP3-KO cells (Figure 3A). The volcano plot presented the

distribution of significant DEGs (Figure 3B). Among the most deregulated genes in ZP3-KO were insulin like growth factor binding protein 4 (IGFBP4), mitochondrially encoded NADH dehydrogenase 2 (MT-ND2), transforming growth factor beta induced (TGFB1), transglutaminase 2 (TGM2) and FOS like 2, AP-1 transcription factor subunit (FOSL2) (upregulated) and tumor protein P53 inducible protein 11 (TP53I11), solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulator 2 (SLC9A3R2), Netrin 4 (NTN4), solute carrier family 16 member 1 (SLC16A), and Ras-related protein Rab-15 (RAB15) (downregulated) (Figure 3C). The GO (gene ontology) enrichment analysis<sup>33</sup> showed that angiogenesis, cell migration and cell adhesion signaling pathways were the most significantly deregulated in A549 ZP3-KO cells versus A549 WT (Figure 3D). Representative DEGs of angiogenesis pathway were the downregulated angiopoietin-like 4 (ANGPTL4) and the upregulated transforming growth factor  $\beta$ -induced protein (TGFB1). DEGs involved in cell migration were the downregulated midkine (MDK) and carcinoembryonic antigen cell adhesion molecule 6 (CEACAM6), as well as the upregulated dual specificity phosphatase 1 (DUSP1). Representative cell adhesion DEGs were the downregulated contactin 1 (CNTN1) and the upregulated protein tyrosine phosphatase receptor type F (PTPRF) (Figure 3D). Additionally, several proteins of the solute carrier family responsible for nutrient transport were deregulated as well (Figure 3D). The representative DEGs from total RNA sequencing were chosen based on reproducible result by qPCR (see Section 3.5).

### 3.4 | ZP3 has a functional implication for lung cancer tumor growth in vivo

To prove the ZP3 effects on lung cancer cells' tumorigenicity in vivo, A549 ZP3-KO and A549 WT cells were inoculated in NOD SCID mice. A significant decrease in tumor growth of A549 ZP3-KO tumors could be observed at every time point after 2, 3, and 4 weeks, compared to the corresponding A549 WT tumors (Figure 4A). Consistent with the tumor volume analysis, the A549 ZP3-KO tumor weights, at autopsy after 4 weeks, were significantly decreased compared to the A549 WT tumors (Figure 4B). Histopathological examinations also revealed significant morphological changes between the A549 ZP3-KO versus WT tumors (Figure 4C-F). The A549 WT tumor showed features of higher malignancy, namely, exhibiting cellular atypia, large nuclei, and a solid pattern of growth with no glandular differentiation

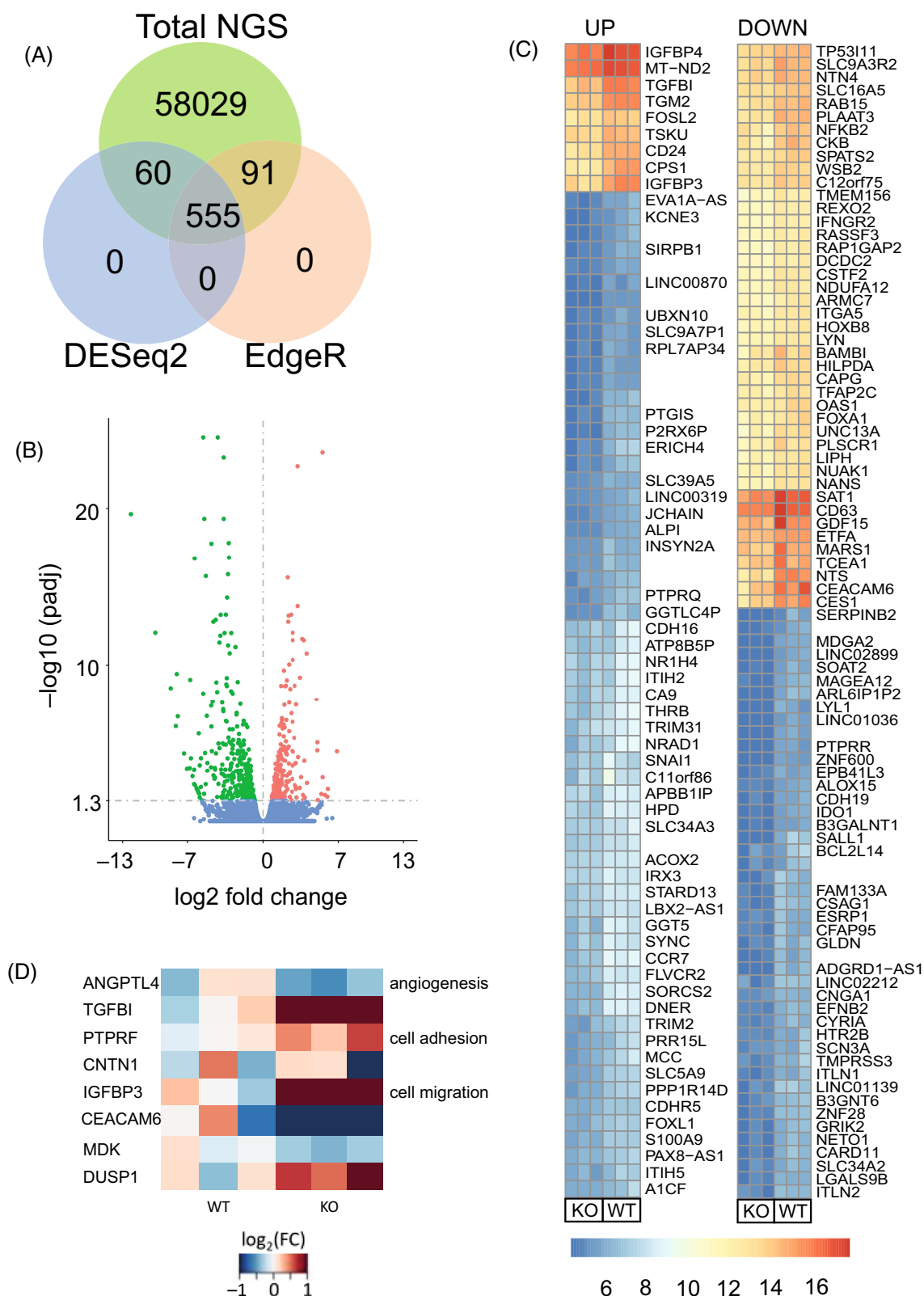
**FIGURE 1** ZP3 expression at protein and mRNA levels in lung cancer tissues and in lung cancer-derived cell lines. Histopathological analyses of lung adenocarcinoma (A), squamous cell lung carcinoma (B) and small cell lung carcinoma (C). Immunohistochemical analyses of the ZP3 staining in lung adenocarcinoma (D), squamous cell lung carcinoma (E) and small cell lung carcinoma (F). RNAscope in situ hybridization using ZP3 probe in lung adenocarcinoma (G), squamous cell lung carcinoma (H) and small cell lung carcinoma (I). qPCR analysis of the ZP3 expression in lung adenocarcinoma, squamous cell lung carcinoma and small cell lung carcinoma relative to peptidylprolyl isomerase A (PPIA) with ovary as a positive tissue control (J). qPCR analysis of the relative ZP3 expression in A549, Calu3, H1975 and H2030 cell lines with ovary as a positive tissue control (K). Each bar represents the mean  $\pm$  SD. Immunohistochemical analysis of the ZP3 staining in the A549 cells (L). Original magnification, 40x; scale bar, 20  $\mu$ m. Black arrowheads indicate positive staining for ZP3. 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; OV, ovary. Original magnification, 40x; scale bar, 20  $\mu$ m.



**FIGURE 2** Knockout of the ZP3 gene effects on A549 cell viability, proliferation and invasion rates. Immunocytochemical analyses of the ZP3 expression in A549 WT (A), A549 ZP3-KO D14 (B) and A549 ZP3-KO D21 (C). qPCR analysis of the ZP3 expression in A549 WT, A549 ZP3-KO D14 and A549 ZP3-KO D21 relative to peptidylprolyl isomerase A (PPIA) with ovary as a positive tissue control (D). Effects of the knockout of the ZP3 gene on the cell viability (E), cell proliferation rate (F) and cell invasion rate (G) in A549 WT, A549 ZP3-KO D14 and A549 ZP3-KO D21 cells. The bar charts are a representation of the percentage of the control (considered as 100%). Each bar represents the mean  $\pm$  SD. Asterisks indicate significant differences between A549 ZP3-KO clones and A549 WT control (\*,  $P < .05$ ; \*\*,  $P < .01$ ; \*\*\*,  $P < .001$ ; \*\*\*\*,  $P < .0001$ ). ND, not detected.

(Figure 4E), whereas the A549 ZP3-KO tumors showed a higher level of cell differentiation with discernible specialized mucus-producing cells and single glandular structures (Figure 4F).

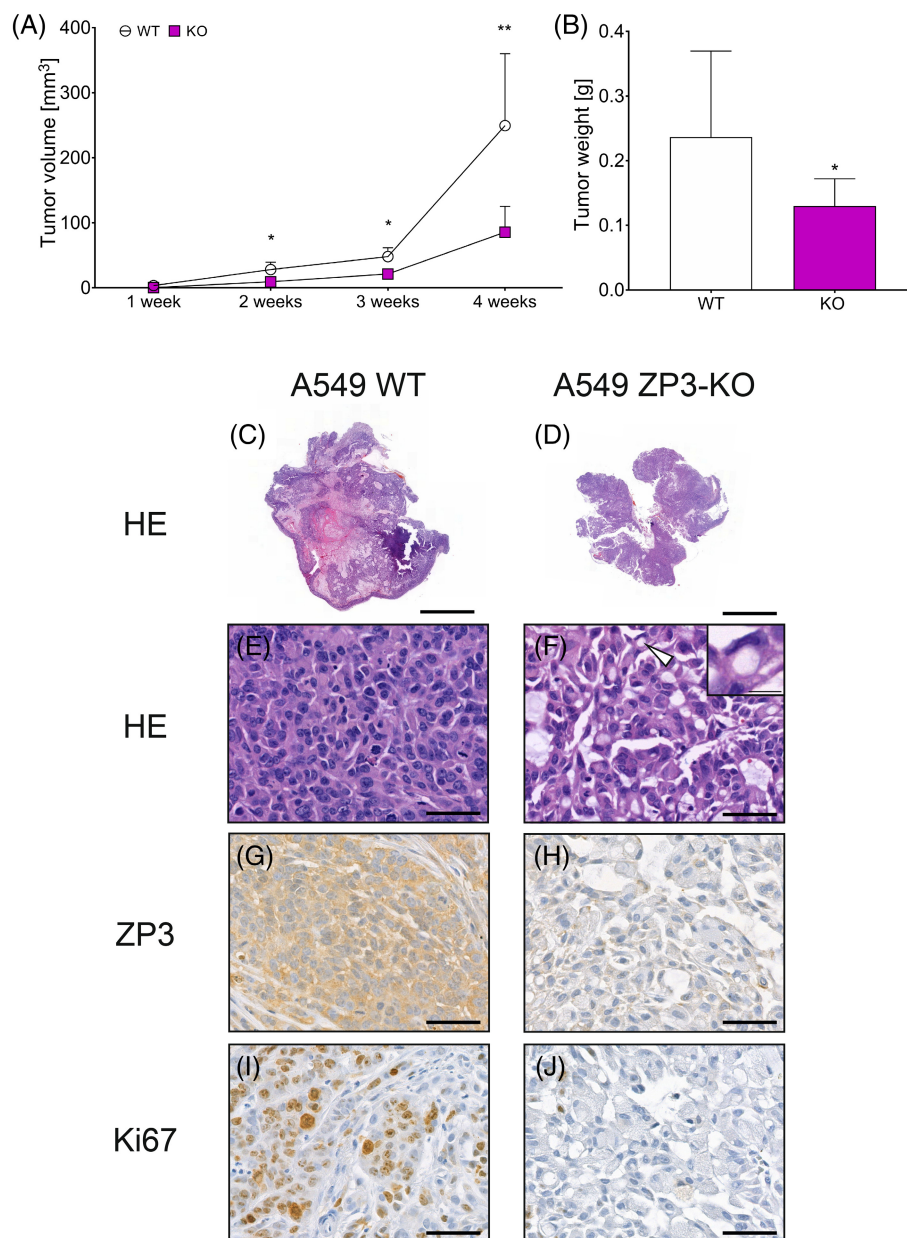
Immunohistochemical analysis showed ZP3 localized abundantly in A549 WT tumors, whereas A549 ZP3-KO tumors were devoid of ZP3 (Figure 4G,H). Additionally, qPCR analysis showed no ZP3 mRNA



**FIGURE 3** Gene expression profile of A549 cells after CRISPR/Cas9 ZP3 gene knockout. The Venn diagram of all detected genes (green) as well as differentially expressed genes detected using the DESeq2 algorithm (blue) and EdgeR algorithm (orange) (A). The volcano plot shows the distribution of gene expression log-fold changes with adjusted  $p$ -values ( $p < 0.05$ ;  $\log_2$  fold change  $> 2$ ) (B). Each gene is represented as a single dot; downregulated genes are marked in green; upregulated genes are marked in red. DESeq2 algorithm analysis of the most up- and downregulated genes in A549 D14 ZP3-KO vs. A549 WT (C). Colors on the heatmap scale indicate the fold change. GO analysis of the genes responsive to the knockout of the ZP3 gene. Heatmaps of the genes related to angiogenesis, cell migration and cell adhesion (D).



**FIGURE 4** In vivo tumor growth of A549 WT and A549 ZP3-KO tumor xenografts. A549 WT and A549 ZP3-KO tumor volumes for 4 weeks (A) and tumor weights upon autopsy (B) in NOD SCID mice. Each bar represents the mean  $\pm$  SD. Asterisks indicate significant differences between A549 ZP3-KO and A549 WT tumors (\*,  $P < .05$ ; \*\*,  $P < .01$ ). Histopathological analyses of the A549 WT (C,E) and A549 ZP3-KO (D,F) tumors. Immunohistochemical analyses of the ZP3 staining of the A549 WT tumor (G) and A549 ZP3-KO (H) tumors. Immunohistochemical analyses of the Ki-67 staining of the A549 WT tumor (I) and A549 ZP3-KO (J) tumors. Original magnification, 40x; scale bar, 20  $\mu$ m.



expression in A549 ZP3-KO tumors (Figure S4). A549 ZP3-KO tumors demonstrated a less abundance of Ki67-positive cells compared to A549 WT tumors (Figure 4I,J), and optical density analysis with Image J showed a significantly decreased percentage of Ki67-positive cells in A549 ZP3-KO tumors (Figure S5). Similarly, no ZP3 was detected in ZP3-KO tumors (Figure S5).

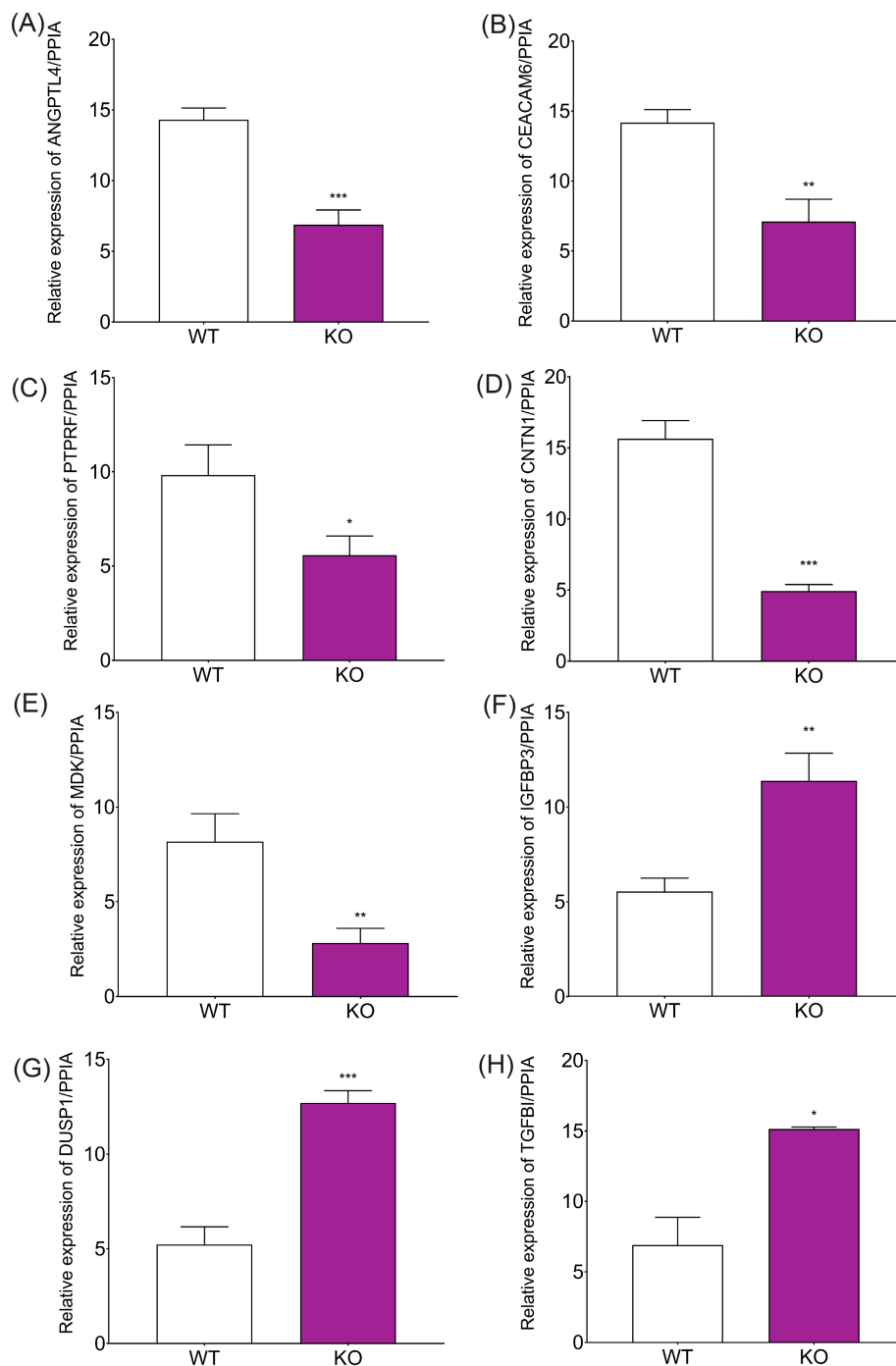
### 3.5 | Validation of the significantly expressed DEGs by qPCR in A549 tumor explants

To validate the RNAseq DEG results from A549 ZP3-KO cells, tumor explants from NOD SCID mice were isolated and qPCR analysis was performed from them. Genes that appeared significant in the GO enrichment analysis were taken into account. Genes *ANGPTL4*

(Figure 5A), *CEACAM6* (Figure 5B), *PTPRF* (Figure 5C), *CNTN1* (Figure 5D) and *MDK* (Figure 5E) were downregulated, whereas *IGFBP3* (Figure 5F), *DUSP1* (Figure 5G) and *TGFBI* (Figure 5H) were upregulated in the A549 ZP3-KO tumor explants. The qPCR results were in line with the RNAseq analysis data.

## 4 | DISCUSSION

In this study, we showed abundant expression of ZP3 in non-small cell lung carcinomas, that is, lung adenocarcinoma and squamous cell carcinoma, as well as, in traceable amounts in small cell lung carcinoma—with a combined ~88% incidence rate. Our functional studies demonstrated that ZP3 knockout in lung adenocarcinoma A549 cells significantly impaired the cell viability, proliferation and



**FIGURE 5** Validation of the selected differentially expressed genes in A549 ZP3-KO tumor xenografts. qPCR analysis of the ANGPTL4 (A), CEACAM6 (B), PTPRF (C), CNTN1 (D), MDK (E), IGFBP3 (F), DUSP1 (G) and TGFBI (H) expression levels in the A549 ZP3-KO vs. A549 WT tumors relative to peptidylprolyl isomerase A (PPIA). Each bar represents the mean  $\pm$  SD relative to PPIA. Asterisks indicate significant differences between A549 ZP3-KO and A549 WT tumors (\*,  $P < .05$ ; \*\*,  $P < .01$ ; \*\*\*,  $P < .001$ ).

invasion rates. Similarly, another glycoprotein of the zona pellucida, ZP2, has been shown to promote cell proliferation in a colon cancer cell line.<sup>34</sup> These findings indicate that ZP proteins may play an important role in cancer biology. Moreover, the A549 ZP3-KO tumors showed transformation toward a more benign phenotype and decreased tumorigenicity in vivo, compared to the WT A549 tumors. A549 WT tumors showed a solid pattern of growth with no glandular differentiation, a characteristic feature of high malignancy that is usually related to poor prognosis in cancer patients.<sup>35</sup> Along with the knockout of ZP3, in A549-KO cells, these malignant features normalized. It could be emphasized the fact that the xenografts were not derived

from biopsies/cancer samples but from an adherent cancer cell line, which was much more homogenous and undifferentiated compared to naturally occurring tumors/cancer tissue. Due to this reason, in monolayer culture and A549 WT xenografts, we observed limited cell differentiation or the formation of clearly defined glandular cells. Similar morphology (phenomenon) can be seen in cell line-derived xenografts in another publication.<sup>36</sup> Therefore, our results implicated an important functional role of ZP3 expression in lung cancer formation and growth.

To gain more in-depth insight into the biological changes triggered by the knockout of ZP3, RNAseq analysis was performed. Several genes

with a known oncogenic function were downregulated in the ZP3-KO cells, such as *ANGPTL4* and *CEACAM6*, known to promote tumor growth and metastases and to correlate with poor prognosis in patients.<sup>37,38</sup> However, one of the upregulated genes was a zinc transporter—*solute carrier family 39 member 5* (*SLC39A5*, also known as *ZIP5*), recently described as a lung cancer promoter gene activating the PI3K/AKT signaling pathway.<sup>39</sup> *SLC39A5* is involved in gene transcription, cell growth and differentiation since the transported zinc is an important cofactor for many enzymes.<sup>40</sup> Not much is yet known about the functional role of *SLC39A5* in tumorigenesis, although while upregulated, it may act as an oncogene in breast and esophageal cancer.<sup>41,42</sup> *SLC39A5* is downregulated in hepatocellular carcinoma.<sup>43</sup> The role of *SLC39A5* is not yet clear in lung adenocarcinoma, and more research is needed.

Other genes from the solute carrier family were also dysregulated in the A549 ZP3-KO cells. Genes such as *SLC16A5* (a lactate and ketone bodies transporter), and *SLC34A3* (a phosphate transporter through sodium cotransport) were downregulated whereas *SLC5A9* (sodium glucose symporter) was upregulated. This may suggest that the overall nutrient transport within the A549 cells has been affected. Notably, in the absence of ZP3 expression, *SLC9A3R2* was significantly downregulated. This protein acts as a scaffold and regulates membrane expression and protein–protein interactions of membrane receptors.<sup>44</sup> This may relate to other changes observed in our A549 ZP3-KO model.

In the RNAseq data GO enrichment analysis, cells devoid of ZP3 expression presented with dysregulated molecular pathways involved in cell adhesion, cell migration and angiogenesis. One of the genes upregulated in the cell adhesion pathways, *PTPRF* was described as a tumor suppressor in breast cancer, and it is tested as a biomarker during NSCLC treatment with Erlotinib.<sup>45,46</sup> However, an adhesion protein *CNTN1* was significantly downregulated. Contactin has been shown as a tumor promoter working through the AKT signaling pathway in lung cancer and several other cancers, such as prostate and breast cancers.<sup>47,48</sup> Its decreased expression has been correlated with a better prognosis.<sup>49</sup> Furthermore, from the genes involved in cell migration, the differentially upregulated genes are described as tumor suppressors in lung adenocarcinoma, for example, *IGFBP3* acting through the IGF1 pathway<sup>50</sup> and the aforementioned *CEACAM6*. The downregulated genes involved in cell migration have been demonstrated to be tumor-promoting agents, such as *MDK* and *DUSP1* involved in the MAPK signaling pathway.<sup>51,52</sup> *MDK* and *DUSP1* are also involved in regulating tumor angiogenesis, alongside other genes identified in this study, like *ANGPTL4* which was downregulated and *TGFBI* upregulated.<sup>53,54</sup> High expression of *ANGPTL4* was described to correlate with poor prognosis in lung cancer patients.<sup>37</sup> On the other hand, high expression of *TGFBI* correlates with a good prognosis for lung cancer patients.<sup>55</sup> Overall, a trend can be noticed where genes related to poor prognoses are downregulated and genes related to better prognoses are upregulated. Taken together, our results suggested that ZP3 may play an important role in the lung adenocarcinoma cell signaling process.

We designed this study to gain an in-depth insight into the functional role of ZP3 in the pathomechanisms of lung cancer. These

findings provide information about the novel biological and functional role of ZP3 in lung cancer. Knockout of ZP3 dysregulated cell viability, proliferation and migration, also tumor formation and growth in vivo, which were closely related to the malignant potential of the cancer tissue. This missing link on the functional implication of ZP3 expression in lung cancer further emphasizes the potential of ZP3 as a target in cancer immunotherapy.

## AUTHOR CONTRIBUTIONS

**Kamila Pulawska-Moon:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; software; validation; visualization; writing – original draft. **Donata Ponikwicka-Tyszk:** Data curation; formal analysis; investigation; methodology; validation; visualization; writing – original draft. **Weronika Lebiecinska:** Data curation; formal analysis; methodology. **Agata Pilaszewicz-Puza:** Data curation; formal analysis; visualization. **Piotr Bernaczyk:** Data curation; formal analysis; methodology; visualization. **Mariusz Koda:** Data curation; methodology; visualization. **Oana Lupu:** Data curation; investigation; methodology. **Gabriela Milewska:** Data curation; investigation; methodology. **Chen-Che Jeff Huang:** Data curation; formal analysis; methodology. **Huifei Zheng:** Data curation; formal analysis; methodology. **Phillip Schiele:** Investigation; methodology. **Il-Kang Na:** Investigation; methodology; validation. **Marco Frentsch:** Data curation; investigation; methodology. **Xiangdong Li:** Data curation; formal analysis; methodology; validation. **Jorma Toppari:** Data curation; funding acquisition; validation. **Slawomir Wolczynski:** Data curation; funding acquisition; project administration; validation. **Herjan J. T. Coelingh Bennink:** Data curation; funding acquisition; investigation. **Ilpo Huhtaniemi:** Conceptualization; data curation; validation; writing – original draft. **Nafis A. Rahman:** Conceptualization; data curation; funding acquisition; investigation; methodology; project administration; supervision; validation; writing – original draft; writing – review and editing.

## FUNDING INFORMATION

This study was partially supported by grants from the Ella and Georg Ehrnrooth Foundation (K.P.-M.), Pantarhei Bioscience/Oncology (N.A.R.), Medical University of Bialystok SUB/1/DN/20/005/1104 (S. W.), Magnus Ehrnrooth Foundation (N.A.R.), and Turku University Hospital (J.T.).

## CONFLICT OF INTEREST STATEMENT

Herjan J. T. Coelingh Bennink is one of the owners of the Pantarhei Bioscience/Oncology. The other authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The raw data of RNA sequencing have been deposited to BioProject, available under accession number PRJNA1101917 in the NCBI BioProject database (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1101917>). The CRISPR/Cas9 ZP3 knockout A549 cell line (ZP3-KO) can be provided upon reasonable request and following approval by the institutional review board of the University of Turku. Other data that support the



findings of this study are available from the corresponding author upon request.

## ETHICS STATEMENT

Written informed consent was obtained from all patients before inclusion. The Medical University of Białystok Local Human Investigation Ethics Committee approved the study (R-I-002/323/2019, APK.002.410.2023). The research was carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki). The Ethics Committee for animal experimentation of the University of Białystok, Białystok, Poland approved the animal experiments (Animal Licence number: 2014/31).

## ORCID

Kamila Pulawska-Moon  <https://orcid.org/0000-0002-9179-0444>

Donata Ponikwicka-Tyszkó  <https://orcid.org/0000-0003-1086-7460>

Weronika Lebieczńska  <https://orcid.org/0000-0002-7661-3757>

Agata Pilaszewicz-Puza  <https://orcid.org/0000-0002-7293-0499>

Piotr Bernaczyk  <https://orcid.org/0000-0003-3401-6599>

Mariusz Koda  <https://orcid.org/0000-0002-1678-567X>

Oana Lupu  <https://orcid.org/0000-0002-1348-7159>

Gabriela Milewska  <https://orcid.org/0000-0001-9008-8285>

Chen-Che Jeff Huang  <https://orcid.org/0000-0003-0376-4682>

Huifei Zheng  <https://orcid.org/0000-0002-7957-7958>

Phillip Schiele  <https://orcid.org/0000-0002-2342-3597>

Il-Kang Na  <https://orcid.org/0000-0001-9902-5424>

Marco Frentsch  <https://orcid.org/0000-0003-3200-210X>

Xiangdong Li  <https://orcid.org/0000-0003-2261-2393>

Jorma Toppari  <https://orcid.org/0000-0003-2228-334X>

Slawomir Wolczynski  <https://orcid.org/0000-0002-7806-1394>

Herjan J. T. Coelingh Bennink  <https://orcid.org/0000-0003-1550-9400>

Ilpo Huhtaniemi  <https://orcid.org/0000-0001-9092-7886>

Nafis A. Rahman  <https://orcid.org/0000-0001-5257-870X>

## REFERENCES

- Lortet-Tieulent J, Soerjomataram I, Ferlay J, Rutherford M, Weiderpass E, Bray F. International trends in lung cancer incidence by histological subtype: adenocarcinoma stabilizing in men but still increasing in women. *Lung Cancer*. 2014;84:13-22.
- Rodríguez-Canales J, Parra-Cuentas E, Wistuba II. Diagnosis and molecular classification of lung cancer. *Cancer Treat Res*. 2016;170:25-46.
- Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68:394-424.
- Weaver BA. How Taxol/paclitaxel kills cancer cells. *Mol Biol Cell*. 2014;25:2677-2681.
- Ruiz-Cordero R, Devine WP. Targeted therapy and checkpoint immunotherapy in lung cancer. *Surg Pathol Clin*. 2020;13:17-33.
- Wassarman PM. Mammalian fertilization: molecular aspects of gamete adhesion, exocytosis, and fusion. *Cell*. 1999;96:175-183.
- Epifano O, Liang LF, Familiari M, Moos MC Jr, Dean J. Coordinate expression of the three zona pellucida genes during mouse oogenesis. *Development*. 1995;121:1947-1956.
- Liang L, Soyal SM, Dean J. F1Galpa, a germ cell specific transcription factor involved in the coordinate expression of the zona pellucida genes. *Development*. 1997;124:4939-4947.
- Hoodbhoy T, Aviles M, Baibakov B, et al. ZP2 and ZP3 traffic independently within oocytes prior to assembly into the extracellular zona pellucida. *Mol Cell Biol*. 2006;26:7991-7998.
- Bleil JD, Wassarman PM. Sperm-egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. *Dev Biol*. 1983;95:317-324.
- Chen T, Bian Y, Liu X, et al. A recurrent missense mutation in ZP3 causes empty follicle syndrome and female infertility. *Am J Hum Genet*. 2017;101:459-465.
- Liu C, Litscher ES, Mortillo S, et al. Targeted disruption of the mZP3 gene results in production of eggs lacking a zona pellucida and infertility in female mice. *Proc Natl Acad Sci U S A*. 1996;93:5431-5436.
- Paterson M, Jennings ZA, van Duin M, Aitken RJ. Immunoneutralization with zona pellucida proteins. *Cells Tissues Organs*. 2000;166:228-232.
- Mahi-Brown CA, Yanagimachi R, Nelson ML, Yanagimachi H, Palumbo N. Ovarian histopathology of bitches immunized with porcine zona pellucida. *Am J Reprod Immunol Microbiol*. 1988;18:94-103.
- Paterson M, Jennings ZA, Wilson MR, Aitken RJ. The contraceptive potential of ZP3 and ZP3 peptides in a primate model. *J Reprod Immunol*. 2002;53:99-107.
- Mohd-Lila MA, Yee LK, Cen LS, et al. The application of naked DNA plasmid (DrZP3) and recombinant adenovirus (Ad-rZP3) in rat animal model to determine comparative efficacy of ZP3-Immunoneutralization vaccines. *Microb Pathog*. 2019;134:103572.
- Pulawska K, Ponikwicka-Tyszkó D, Lebieczńska W, et al. Novel expression of zona pellucida 3 protein in normal testis; potential functional implications. *Mol Cell Endocrinol*. 2022;539:111502.
- Rahman NA, Bennink HJ, Chrusciel M, et al. A novel treatment strategy for ovarian cancer based on immunization against zona pellucida protein (ZP) 3. *FASEB J*. 2012;26:324-333.
- Chrusciel M, Doroszko M, Stelmazewska J, et al. Transgenic mice expressing inhibin alpha-subunit promoter (inhalpha)/Simian Virus 40 T-antigen (Tag) transgene as a model for the therapy of granulosa cell-derived ovarian cancer. *Reprod Biol*. 2014;14:25-31.
- Costa J, Pereira R, Oliveira J, et al. Structural and molecular analysis of the cancer prostate cell line PC3: oocyte zona pellucida glycoproteins. *Tissue Cell*. 2018;55:91-106.
- Coelingh Bennink HJT. *Immunotherapeutic Methods for Treating and/or Preventing Lung Cancer*. Pantarhei Bioscience BV Z; 2019.
- Bennink HJT. *Immunotherapeutic Method for Treating Prostate Cancer*. Pantarhei Bioscience BV Z; 2016.
- Buschmann TFN-K, Fuchs M, Heim S, et al. Inventor. Differentially Expressed Tumour-Specific Polypeptides for Use in the Diagnosis and Treatment of Cancer, CA Patent CA2539490A1; 2004.
- Bi Y, Jin S, Tang G, et al. Prognostic significance of ZP3 in hepatocellular carcinoma. *Comb Chem High Throughput Screen*. 2023;26:1729-1736.
- Bertozzi S, Londero AP, Xholli A, et al. Risk-reducing breast and gynecological surgery for BRCA mutation carriers: a narrative review. *J Clin Med*. 2023;12:12.
- Domchek SM, Friebel TM, Singer CF, et al. Association of risk-reducing surgery in BRCA1 or BRCA2 mutation carriers with cancer risk and mortality. *Jama*. 2010;304:967-975.
- Jacoby VL, Grady D, Wactawski-Wende J, et al. Oophorectomy vs ovarian conservation with hysterectomy: cardiovascular disease, hip fracture, and cancer in the Women's Health Initiative Observational Study. *Arch Intern Med*. 2011;171:760-768.

28. Marchetti C, De Felice F, Palaia I, et al. Risk-reducing salpingo-oophorectomy: a meta-analysis on impact on ovarian cancer risk and all cause mortality in BRCA 1 and BRCA 2 mutation carriers. *BMC Womens Health*. 2014;14:150.
29. Tsai CM, Chang KT, Wu LH, et al. Correlations between intrinsic chemoresistance and HER-2/neu gene expression, p53 gene mutations, and cell proliferation characteristics in non-small cell lung cancer cell lines. *Cancer Res*. 1996;56:206-209.
30. NCI-Navy Medical Oncology Branch cell line supplement. *J Cell Biochem Suppl*. 1996;24:1-291.
31. Giard DJ, Aaronson SA, Todaro GJ, et al. In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J Natl Cancer Inst*. 1973;51:1417-1423.
32. Shen BQ, Finkbeiner WE, Wine JJ, Mrsny RJ, Widdicombe JH. Calu-3: a human airway epithelial cell line that shows cAMP-dependent Cl-secretion. *Am J Physiol*. 1994;266:L493-L501.
33. Mi H, Muruganujan A, Ebert D, Huang X, Thomas PD. PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. *Nucleic Acids Res*. 2019;47:D419-D426.
34. Kraus D, Glassmann A, Golletz C, Kristiansen G, Winter J, Probstmeier R. Zona pellucida protein 2 (ZP2) is expressed in colon cancer and promotes cell proliferation. *Cancers (Basel)*. 2021;13(8):1759.
35. Miyahara N, Nii K, Benazzo A, et al. Solid predominant subtype in lung adenocarcinoma is related to poor prognosis after surgical resection: a systematic review and meta-analysis. *Eur J Surg Oncol*. 2019;45:1156-1162.
36. Lei X, Li Z, Huang M, et al. Gli1-mediated tumor cell-derived bFGF promotes tumor angiogenesis and pericyte coverage in non-small cell lung cancer. *J Exp Clin Cancer Res*. 2024;43:83.
37. Zhang L, Zhang Z, Yu Z. Identification of a novel glycolysis-related gene signature for predicting metastasis and survival in patients with lung adenocarcinoma. *J Transl Med*. 2019;17:423.
38. Beauchemin N, Arabzadeh A. Carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) in cancer progression and metastasis. *Cancer Metastasis Rev*. 2013;32:643-671.
39. Liu Z, Hu Z, Cai X, Liu S. SLC39A5 promotes lung adenocarcinoma cell proliferation by activating PI3K/AKT signaling. *Pathol Res Pract*. 2021;224:153541.
40. Lichten LA, Cousins RJ. Mammalian zinc transporters: nutritional and physiologic regulation. *Annu Rev Nutr*. 2009;29:153-176.
41. Liu L, Yang J, Wang C. Analysis of the prognostic significance of solute carrier (SLC) family 39 genes in breast cancer. *Biosci Rep*. 2020;40(8):BSR20200764.
42. Jin J, Li Z, Liu J, Wu Y, Gao X, He Y. Knockdown of zinc transporter ZIP5 (SLC39A5) expression significantly inhibits human esophageal cancer progression. *Oncol Rep*. 2015;34:1431-1439.
43. Bitirim CV. The role of zinc transporter proteins as predictive and prognostic biomarkers of hepatocellular cancer. *PeerJ*. 2021;9:e12314.
44. He P, Zhang H, Yun CC. IRBIT, inositol 1,4,5-triphosphate (IP3) receptor-binding protein released with IP3, binds Na<sup>+</sup>/H<sup>+</sup> exchanger NHE3 and activates NHE3 activity in response to calcium. *J Biol Chem*. 2008;283:33544-33553.
45. Soulieres D, Hirsch FR, Shepherd FA, et al. PTPRF expression as a potential prognostic/predictive marker for treatment with erlotinib in non-small-cell lung cancer. *J Thorac Oncol*. 2015;10:1364-1369.
46. Xu YY, Liu H, Su L, et al. PPARgamma inhibits breast cancer progression by upregulating PTPRF expression. *Eur Rev Med Pharmacol Sci*. 2019;23:9965-9977.
47. Yan J, Ojo D, Kapoor A, et al. Neural cell adhesion protein CNTN1 promotes the metastatic progression of prostate cancer. *Cancer Res*. 2016;76:1603-1614.
48. Chen N, He S, Geng J, et al. Overexpression of Contactin 1 promotes growth, migration and invasion in Hs578T breast cancer cells. *BMC Cell Biol*. 2018;19:5.
49. Yan J, Wong N, Hung C, Chen WX, Tang D. Contactin-1 reduces E-cadherin expression via activating AKT in lung cancer. *PLoS One*. 2013;8:e65463.
50. Kuhn H, Frille A, Petersen MA, et al. IGFBP3 inhibits tumor growth and invasion of lung cancer cells and is associated with improved survival in lung cancer patients. *Transl Oncol*. 2023;27:101566.
51. Yuan K, Chen Z, Li W, et al. MDK protein overexpression correlates with the malignant status and prognosis of non-small cell lung cancer. *Arch Med Res*. 2015;46:635-641.
52. Moncho-Amor V, Ibanez de Caceres I, Bandres E, et al. DUSP1/MKP1 promotes angiogenesis, invasion and metastasis in non-small-cell lung cancer. *Oncogene*. 2011;30:668-678.
53. Wen G, Partridge MA, Li B, et al. TGFBI expression reduces in vitro and in vivo metastatic potential of lung and breast tumor cells. *Cancer Lett*. 2011;308:23-32.
54. Fernandez-Hernando C, Suarez Y. ANGPTL4: a multifunctional protein involved in metabolism and vascular homeostasis. *Curr Opin Hematol*. 2020;27:206-213.
55. Pajares MJ, Agorreta J, Salvo E, et al. TGFBI expression is an independent predictor of survival in adjuvant-treated lung squamous cell carcinoma patients. *Br J Cancer*. 2014;110:1545-1551.

## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Pulawska-Moon K, Ponikwicka-Tyszko D, Lebiecinska W, et al. Novel ectopic expression of zona pellucida 3 glycoprotein in lung cancer promotes tumor growth. *Int J Cancer*. 2024;155(10):1846-1857. doi:10.1002/ijc.35098