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Inhibition of the Cyclin K-CDK12 complex induces DNA damage and increases the effect of androgen deprivation therapy in prostate cancer

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Abstract

Androgen deprivation therapy (ADT) is the mainstay of the current first-line treatment concepts for patients with advanced prostate carcinoma (PCa). However, due to treatment failure and recurrence investigation of new targeted therapeutics is urgently needed. In this study, we investigated the suitability of the Cyclin K-CDK12 complex as a novel therapeutic approach in PCa using the new covalent CDK12/13 inhibitor THZ531. Here we show that THZ531 impairs cellular proliferation, induces apoptosis, and decreases the expression of selected DNA repair genes in PCa cell lines, which is associated with an increasing extent of DNA damage. Furthermore, combination of THZ531 and ADT leads to an increase in these anti-tumoral effects in androgen-sensitive PCa cells. The anti-proliferative and pro-apoptotic activity of THZ531 in combination with ADT was validated in an ex vivo PCa tissue culture model. In a retrospective immunohistochemical analysis of 300 clinical tissue samples we show that Cyclin K (CycK) but not CDK12 expression correlates with a more aggressive type of PCa. In conclusion, this study demonstrates the clinical relevance of the CycK-CDK12 complex as a promising target for combinational therapy with ADT in PCa and its importance as a prognostic biomarker for patients with PCa.

KEYWORDS
androgen deprivation therapy, Cyclin K-CDK12 complex, DNA damage, prostate cancer, THZ531

Abbreviations: ADT, androgen deprivation therapy; AR, androgen receptor; CAM, chicken chorioallantoic membrane; CDK12/13, cyclin-dependent kinase 12 and 13; CRPC, castration-resistant prostate cancer; CTD, C-terminal domain; CycK, Cyclin K; DDR, DNA damage response; H&E, hematoxylin and eosin; HR, homologous recombination; mCRPC, metastatic castration-resistant prostate cancer; PARP, poly (ADP-ribose) polymerase; PCa, prostate carcinoma; PIN, prostatic intraepithelial neoplasia; PSA, prostate specific antigen; PTEN, phosphatase and tensin homolog; RNAP II, RNA polymerase II; TMA, tissue microarray.

What's new?

The recurrence of prostate cancer (PCa) following androgen deprivation therapy (ADT) is associated with treatment resistance. A promising avenue for overcoming resistance involves targeted inhibition of CycK-CDK12, a complex that serves a critical role in PCa cell survival. The present study, using an ex vivo PCa tissue culture model, shows that THZ531, a covalent CDK12/13 inhibitor, increases the responsiveness of androgen-sensitive PCa cells to ADT and exerts antiproliferative, pro-apoptotic activity. Meanwhile, more aggressive PCa was correlated with CycK expression. The findings highlight the clinical relevance of CDK12 inhibition and CycK expression, opening new prognostic and therapeutic opportunities for PCa.

1 | INTRODUCTION

PCa is, with 27% of newly diagnosed cases, the most common diagnosed cancer and the second leading cause of cancer-related death in men worldwide.[1] The initial management of localized PCa may be active surveillance, focal therapy, radical prostatectomy or radiotherapy, which is combined with ADT in some cases.[2] Metastatic disease is initially treated by combining ADT with docetaxel and/or novel hormonal agents.[3] Various molecular alterations contribute to the heterogeneity and aggressiveness of PCa, including chromosomal alterations, epigenetic mutations in targetable pathways (eg, DNA repair), and mutations or splice variants of the androgen receptor (AR).[4] The persistent transcriptional activity of the AR plays a central role in the progression to castration-resistant PCa (CRPC) or metastatic (m) CRPC.[5] Patients with this type of advanced PCa continue on ADT with for example, the AR inhibitor enzalutamide or abiraterone acetate, a selective CYP17 inhibitor, which suppresses the androgen synthesis in testes, adrenal glands, and prostate tissue.[5] Further treatment options for mCRPC include cytotoxic therapy with taxanes, radium-223, lutetium-(177)-prostate-specific membrane antigen-radioligand therapy and olaparib in males with homologous recombination (HR) deficiency.[6] The development of aggressive cancer variants is characterized by resistance to therapy and the lack of clinically relevant biomarkers remain key challenges for treating PCa, so new strategies are urgently needed.

Cyclin-dependent kinases (CDKs) are serine/threonine kinases that can be divided into two classes: cell cycle-associated CDKs (CDK1, CDK2, CDK4 and CDK6) or transcription-associated CDKs (CDK7, CDK8, CDK9, CDK12 and CDK13).[7] CDK12 and its homolog CDK13 form two separate complexes with the regulatory subunit CycK and promote transcription elongation by phosphorylating Ser2, Ser5 or Ser7 residues in the C-terminal domain (CTD) of RNA Polymerase II (RNAP II).[7-9] In this context, the focus of targeted cancer research is more on the CycK-CDK12 complex, as it regulates cellular processes to maintain genomic stability.[7,8] Furthermore, studies have shown that the absence or silencing of this CycK-CDK12 complex influences the transcription of several DNA damage response (DDR) and DNA repair genes involved in HR[9-11] and reduces cell proliferation in cancer cells.[8,12,13] Depletion or loss of function of the CycK-CDK12 complex sensitizes ovarian cancer cells to DNA-damaging agents or poly (ADP-ribose) polymerase (PARP) inhibitors by causing DNA damage.[8,14,15] The development of the covalent CDK12/13 inhibitor THZ531 brings the CycK-CDK12 complex further into focus as a new promising target in cancer therapy.[10] In a recent article by Lei et al.,[16] CDK12 was identified as essential for PCa cell survival and the inhibition of CDK12 by THZ531 suppressed AR signaling. Furthermore, patients harboring mutated CDK12 exhibit a more aggressive tumor type with poor response to therapy, characterized by genomic instability.[17,18] Thus, further investigation on the suitability of the CycK-CDK12 complex as a novel therapeutic target of PCa or as a biomarker is warranted.

In the present study, we confirmed that inhibition of the CycK-CDK12 complex in PCa cells impairs cell proliferation, induces apoptosis, and decreases the expression of the selected DNA repair genes, which is associated with an increasing amount of DNA damage. Furthermore, we demonstrate a synergistic effect between THZ531 and ADT treatment in androgen-sensitive PCa cells, which was verified in vital human PCa tissue. In addition, we show that CycK expression correlates with a more aggressive PCa type. Here, we demonstrate the rationale for the clinical relevance of inhibiting the CycK-CDK12 complex as a promising target for combination therapy with ADT and its importance as a prognostic biomarker in PCa.

2 | METHODS

2.1 | Cell culture

Five different human PCa cell lines were used: The androgen-irresponsive DU145 and PC-3 cell lines, the androgen-dependent LAPC-4 cell line (wild type AR), the androgen-responsive LNCaP cell line (mutant AR), and its derivative LNCaP C4-2B (mutant AR).[19,20] The PCa cell lines DU145 (RRID: CVCL_0105), LNCaP (RRID: CVCL_0395), PC-3 (RRID: CVCL_0035) and LNCaP C4-2B (RRID: CVCL_4784) were obtained from the American Type Culture Collection (ATCC, USA). The cell line LAPC-4 (RRID: CVCL_4744) was a gift from Dr. A. Cato (University of Karlsruhe, Germany). All cell lines except LNCaP C4-2B were cultured in RPMI-1640 medium (Life Technology Darmstadt, Germany, 21875-034), while LNCaP C4-2B cells were cultured in Dulbecco's modified Eagle medium (Sigma-Aldrich, Darmstadt, Germany, D5671) under standard conditions (37°C and 5% CO_2). All standard medium was supplemented with

10% fetal calf serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Life Technology). For the cultivation of the androgen-dependent LAPC-4 cell line, the medium was supplemented with 1 nM of the synthetic androgen R1881 (Sigma-Aldrich, R0908) dissolved in DMSO. All cell lines were authenticated using short tandem repeat (STR) profiling within the last 3 years (Cell Authentication Service of Eurofins Genomics, Ebersberg, Germany). All experiments were performed with mycoplasma-free cells.

2.2 | Treatment of tumor cells, viability assay, flow cytometry analysis, immunoblot analysis, qRT-PCR analysis, immunofluorescence staining, immunohistochemistry and statistical analysis

A detailed description can be found in the Data S1, Tables S1 and S2.

2.3 | Ex vivo tissue slice culture

Twelve patients diagnosed with a PCa, who underwent prostatectomy at the Department of Urology and Pediatric Urology of the University Medical Center Mainz from 2020 to 2023, were included in the study. The tissue slice culture protocol was carried out as previously described.[21] Fresh prostate tumor tissue was cut into 300 µm thick slices using a vibratome VT1200 (Leica Microsystems, Mannheim, Germany) equipped with a razor blade (Wilkinson Sword, Solingen, Germany). From each tumor punch, the first and last slice was immediately fixated in 4% buffered formalin. The remaining slices were randomized and allocated to control and treatment groups. Tissue slices were cultured on cell culture inserts (Corning, Kaiserslautern, Germany 353090) in six-well plates (Corning, 353502) with Dulbecco's modified Eagle's medium (ATCC, Manassas, FL, 30-2002) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. The plates were incubated under standard conditions (37°C and 5% CO_2) on an orbital shaker (Thermo Scientific, MaxQ2000 CO_2 Plus, 55 rpm). The medium was changed after 1 h and subsequently every additional 24 h the slices were treated as indicated. After 72 h, tissue slices were fixated in 4% buffered formalin, paraffin-embedded, and stained immunohistochemically.

2.4 | Tissue microarray

For the tissue microarray (TMA), tissue samples of 300 patients diagnosed with PCa were provided by the Tissue Biobank of the University Medical Center Mainz. All tumor samples were obtained from patients who underwent radical prostatectomy between 2015 and 2018 and who had not received neoadjuvant therapy. All samples were graded and staged according to the new ISUP 2014/WHO2016 grading system at the Institute of Pathology at the University Medical Center Mainz.[22] The TMA contained 1200 primary tumor samples from two different but morphologically representative tissue regions of 300 patients with PCa. For further analysis, clinicopathological data were available, including Gleason score, grading group, pT and pN stage, and perineural invasion (Pn). Immunohistochemical staining of the TMA slides with anti-CycK and anti-CDK12 was performed as described. After slide digitalization with a slide scanner (NanoZoomer 2.0HT, Hamamatsu Photonics K.K., Japan) staining were analyzed with the open-source digital image software QuPath. To evaluate CycK and CDK12 expression, intensity thresholds for both staining were manually set to categorize tumor cells into negative, weak, moderate or strongly positive. The staining intensity of each tissue core was calculated as an H-score (1× [% tumor cells weakly stained] + 2× [% tumor cells moderately stained] + 3× [% tumor cells strongly stained]), ranging from 0 to 300.

2.5 | Chicken chorioallantoic membrane assay

The chicken chorioallantoic membrane (CAM) model was performed as previously described[23] with the following modifications: Freshly laid fertilized eggs were purchased from VALO Biomedia (Osterholz-Scharmbeck, Germany) and incubated in a digital motor breeder (Easy 250, J. Hemel Brutgeräte, Verl-Kaunitz, Germany). On the fourth day of embryonic development, the eggshell was incised and then sealed with Leukosilk strips in preparation for subsequent tumor implantation. On the seventh day of embryonic development, the CAM was scratched and then small elastic latex rings (DENTAURUM, Ispringen, Germany) were placed on the CAM. Subsequently, 6×10^6 DU145 cells/egg in 25 µl Matrigel Matrix (Corning, 356234) were added onto the rings. On days 11 and 13, eggs were treated with 200 nM THZ531 or with a corresponding control solution. On day 18, the embryos were euthanized with 50 mg/ml ketamine (hameln pharma, Hameln, Germany) per egg. The tumors were resected and fixated in 4% buffered formalin, paraffin-embedded, and stained immunohistochemically. For the analysis of cellular viability, vital and necrotic tumor areas were quantified using H&E-stained sections.

3 | RESULTS

3.1 | Inhibition of the CycK-CDK12 complex induces anti-tumorigenic effects in PCa cells

Previous studies have highlighted the importance of the CycK-CDK12 complex as a druggable target for cancer research as CDK12 has been identified as an oncogenic driver in several malignancies including breast cancer, gastric cancer, and hepatocellular carcinoma.[8,12,16] To investigate the functional role of this complex in PCa cells, we used THZ531, a selective inhibitor of the kinases CDK12 and CDK13.[10] First, we analyzed the endogenous expression levels of CycK, CDK12 and CDK13 in five human PCa cell lines DU145, PC-3, LNCaP C4-2B, LAPC-4 and LNCaP. All cell lines examined showed CycK as well as CDK12 and CDK13 expression (Figure 1A). Treatment with THZ531 resulted in an alteration of CycK-CDK12-mediated phosphorylation

of pSer2/5/7 in the CTD of the RNAP II in all PCa cell lines. In this context, the inhibition of phosphorylation at Ser2 was particularly evident (Figure S1). Functional effects of THZ531 were initially examined by measuring cell viability. With increasing concentrations of THZ531, all cell lines displayed a significant reduction in cell number with LNCaP cells showing only low sensitivity (Figure 1B). To investigate whether this reduction was associated with apoptosis, Annexin V-/7AAD staining and subsequent FACS analysis were performed. THZ531 distinctly induced apoptosis in DU145 and LNCaP C4-2B cells (Figure 1C). Consistently, cleavage of caspase-3 could be

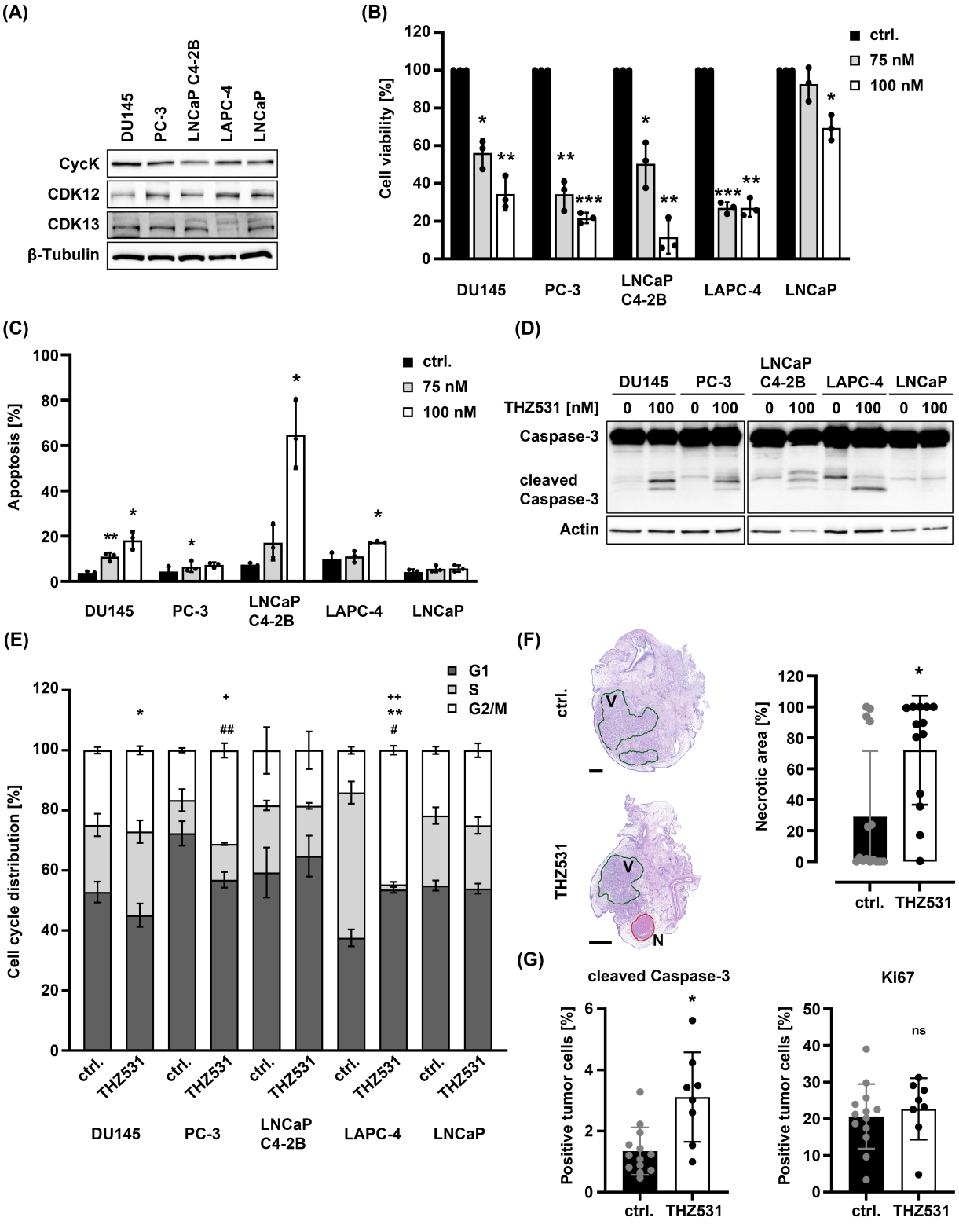

FIGURE 1 Legend on next page.

observed in both cell lines as well as in PC-3 cells (Figure 1D). In contrast, LNCaP and LAPC-4 cells showed no induction of apoptosis. Next, we examined whether changes in cell cycle distribution underlie THZ531-induced cell count reduction. PC-3 and LAPC-4 cells showed a significant increase in the G2/M phase as well as a decrease in G1 phase cells upon THZ531 treatment, accompanied by a significant decrease in S phase in LAPC-4 cells, indicating an inhibition of cellular proliferation (Figure 1E). Further, we evaluated the effects of THZ531 treatment in an avian model. Therefore, DU145 cells were implanted onto the CAM and treated twice with THZ531. In line with our in vitro experiments, we observed increased amounts of cell death upon THZ531 treatment (Figures 1F and S2A), which was accompanied by elevated levels of caspase-3 cleavage (Figures 1G and S2B). No significant differences in the amount of Ki67-positivity in response to THZ531 was observed (Figures 1G and S2C).

3.2 | Inhibition of the CycK-CDK12 complex affects the transcription of DDR genes and causes DNA damage

Former studies have shown that the CycK-CDK12 complex plays an essential role in regulating the transcription of DDR genes by phosphorylating the CTD of RNAP II.[9,10] Subject to this regulation are the expression of several genes involved in HR for example, BRCA1, FANCI, FANCD2 and ATR. An altered expression of these core DDR genes can lead to the accumulation of DNA damage.[8,10,15,24] Based on this, we expected that CycK-CDK12 inhibition would have similar effects in PCa cells. To test this hypothesis, we treated the PCa cell lines with THZ531, followed by an analysis of mRNA and protein levels of eight different DDR genes by qRT-PCR and immunoblot analysis. A CycK-CDK12-dependent regulation of these selected DDR genes has already be shown in previous studies.[8,25] Inhibition of CycK-CDK12 resulted in significantly lower mRNA and protein levels for most of the selected DDR genes in all cell lines except LNCaP cells (Figure 2A-F). In addition, inhibition of the CycK-CDK12 complex resulted in DNA damage (Figures 2G,H and S3). Since we observed a THZ531-dependent decrease in BRCA1 expression and an accumulation of DNA damage, indicating a defective HR, we further investigated the effects of a combined treatment with olaparib and THZ531. The PARP1 inhibitor olaparib inhibits DNA single-strand repair and causes replication-induced DNA damage. Thus, especially cancer cells lacking BRCA1 show an increased sensitivity to olaparib.[14] PCa cells were treated with THZ531, olaparib, and a combination thereof. Subsequent analysis of cell death showed that combined treatment synergistically induced apoptosis in LNCaP cells (Figure S4). In addition, DU145 and LNCaP C4-2B cells showed a higher apoptosis rate in the combined treatment compared to single treatment (Figure S4).

3.3 | Combination of CycK-CDK12 complex inhibition and hormone therapy increases apoptosis and DNA damage in androgen-sensitive cells

Next, we examined the effects of a combined therapy of ADT and CycK-CDK12 inhibition. Importantly, several links between AR signaling and DDR, which also regulates the expression of DNA repair genes in PCa, have been demonstrated recently.[4,26] The different endogenous AR and prostate specific antigen (PSA) expression levels are illustrated in Figure 3A. To investigate the effect of the combination treatment, we treated the androgen-sensitive PCa cell lines simultaneously with THZ531 and enzalutamide or abiraterone. Reduction of PSA expression levels after treatment with enzalutamide or abiraterone confirmed successful inhibition of AR signaling (Figures S5 and 3D). Cell death analysis indicated an increased apoptosis rate in the combined treatment with THZ531 plus enzalutamide or abiraterone compared to the single treatment in LNCaP C4-2B and LAPC-4 cells. Interestingly, the combined treatment also caused apoptosis induction in LNCaP cells (Figure 3B, C). Consistently, cleavage of caspase-3 verified the induction of apoptosis upon treatment combination (Figure 3D). Gene expression analysis of several DDR genes after simultaneous CycK-CDK12 and AR inhibition with enzalutamide

FIGURE 1 THZ531 induces anti-tumorigenic effects in vitro and in vivo. (A) Immunoblot analysis of endogenous CycK, CDK12 and CDK13 expression in several human PCa cell lines. (B-E) PCa cells were treated with 75 and 100 nM of THZ531, respectively. (B) To assess viability, cell number was determined 72 h after treatment. Data represent the mean of three independent experiments (n = 3 ± SD, paired two-tailed *t*-test, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$). (C) After 72 h of treatment, cells were stained with 7-AAD and Annexin V, and apoptosis rate was detected with subsequent FACS analysis. Data represent the mean of three independent experiments (n = 3 ± SD, paired two-tailed *t* test, $^{*}P < .05$, $^{**}P < .01$). (D) Immunoblot analysis for full-length (p32) and cleaved caspase-3 (p19/p17) was performed 72 h after treatment. (E) Analysis of cell cycle distribution after treatment with 100 nM THZ531 was examined after 72 h by FACS analysis. Cells were stained with BrdU for 30 min, followed by simultaneous staining with a FITC-conjugated anti-BrdU antibody and total DNA staining with 7-AAD. Data represent the mean of three independent experiments (n = 3 ± SD, paired two-tailed *t* test, denoting a significant difference in a relative amount of cells in G1, S, and G2/M phase compared to control cells, G1 ($^{\#}P < .05$, $^{\#\#}P < .01$), S ($^{*}P < .05$, $^{**}P < .01$) and G2/M ($^{+}P < .05$, $^{++}P < .01$). (F-G) DU145 cells were transplanted on the CAM of fertilized chicken eggs. Avian xenografts were treated twice with 200 nM THZ531 at day 11 and 13 of embryonic development. (F) Left panel: Representative images of control and THZ531-treated resected xenograft tumors (H&E staining; vital tumor cells (V) are highlighted in green; necrotic tumor cells (N) are highlighted in red; scale bar = 500 µm). Right panel: Data represent the quantification of necrotic tumor area in relation to total resected xenograft tumor (ctrl.: n = 15; THZ531: n = 13 ± SD, Mann-Whitney *U* test, $^{*}P < .05$). (G) Immunohistochemical staining of cleaved caspase-3 (apoptosis) and Ki67 (proliferation) were conducted and analyzed by digital image analysis. Data represent the quantification of the fraction of cleaved caspase-3-positive tumor cells (left panel) and Ki67 positive-tumor cells (right panel) (ctrl.: n = 13, THZ531: n = 8 ± SD, unpaired Welch's *t* test, $^{*}P < .05$; ns, non-significant).

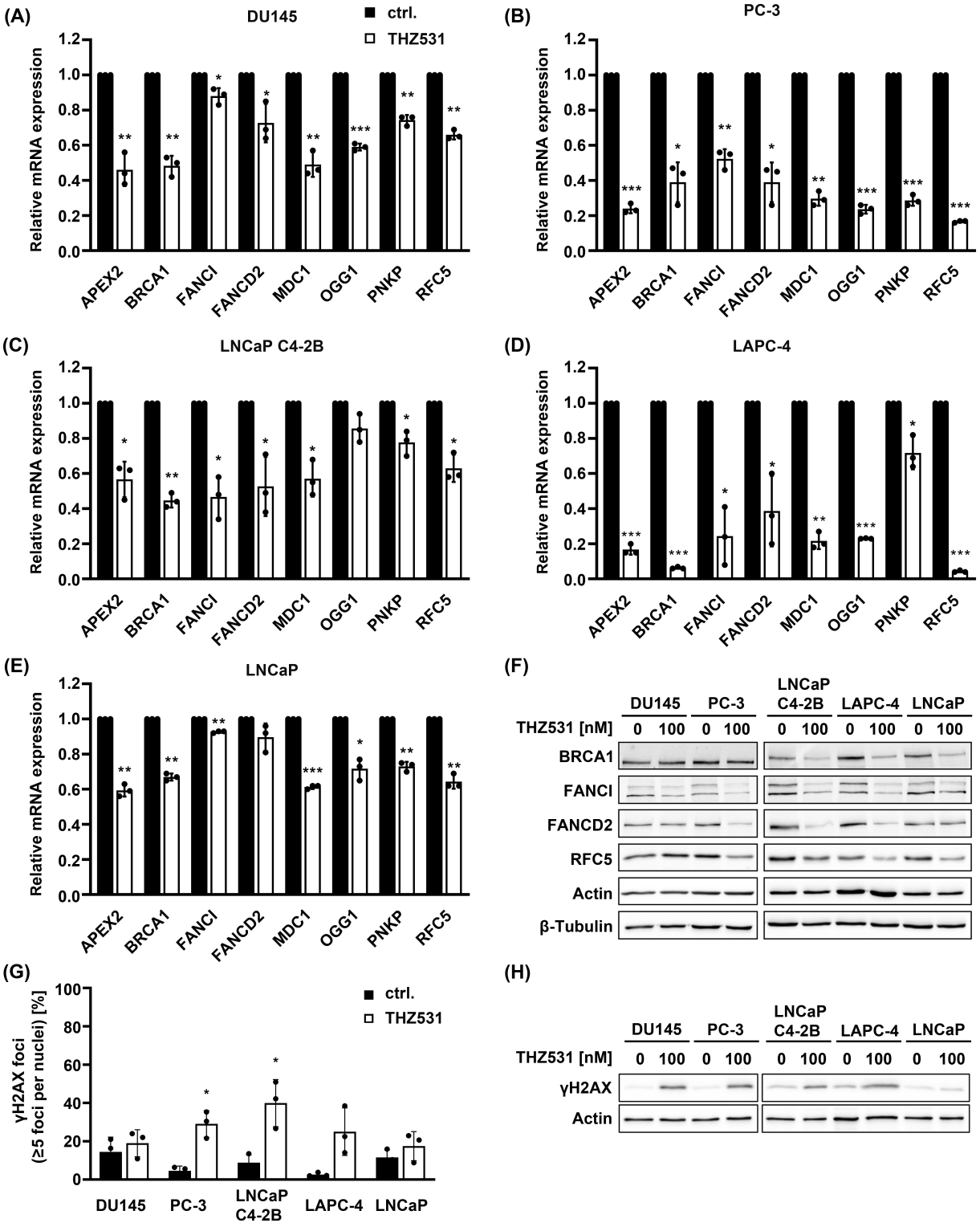

FIGURE 2 CycK-CDK12 complex regulates expression of DNA repair genes and DNA damage repair in PCa cells. (A-H) PCa cells were treated with 100 nM of THZ531. (A-E) RNA was isolated 24 h after treatment and mRNA levels of selected DNA repair genes were determined by qRT-PCR and normalized to internal 18S rRNA levels. Data represent the mean of three independent experiments (n = 3 ± SD, paired two-tailed *t* test, **P* < .05, ***P* < .01, ****P* < .001). (F) 72 h after treatment, whole cell lysates were prepared. Immunoblot analysis of selected proteins involved in DNA repair was performed. (G) Immunofluorescent staining of PCa cells with an anti-γH2AX antibody with subsequent analysis was determined 72 h post treatment. The percentage of ≥5 γH2AX foci per nucleus were quantified. Data represent the mean of three independent experiments (n = 3 ± SD, paired two-tailed *t* test, **P* < .05). (H) Immunoblot analysis of γH2AX protein expression was performed 72 h post treatment with THZ531.

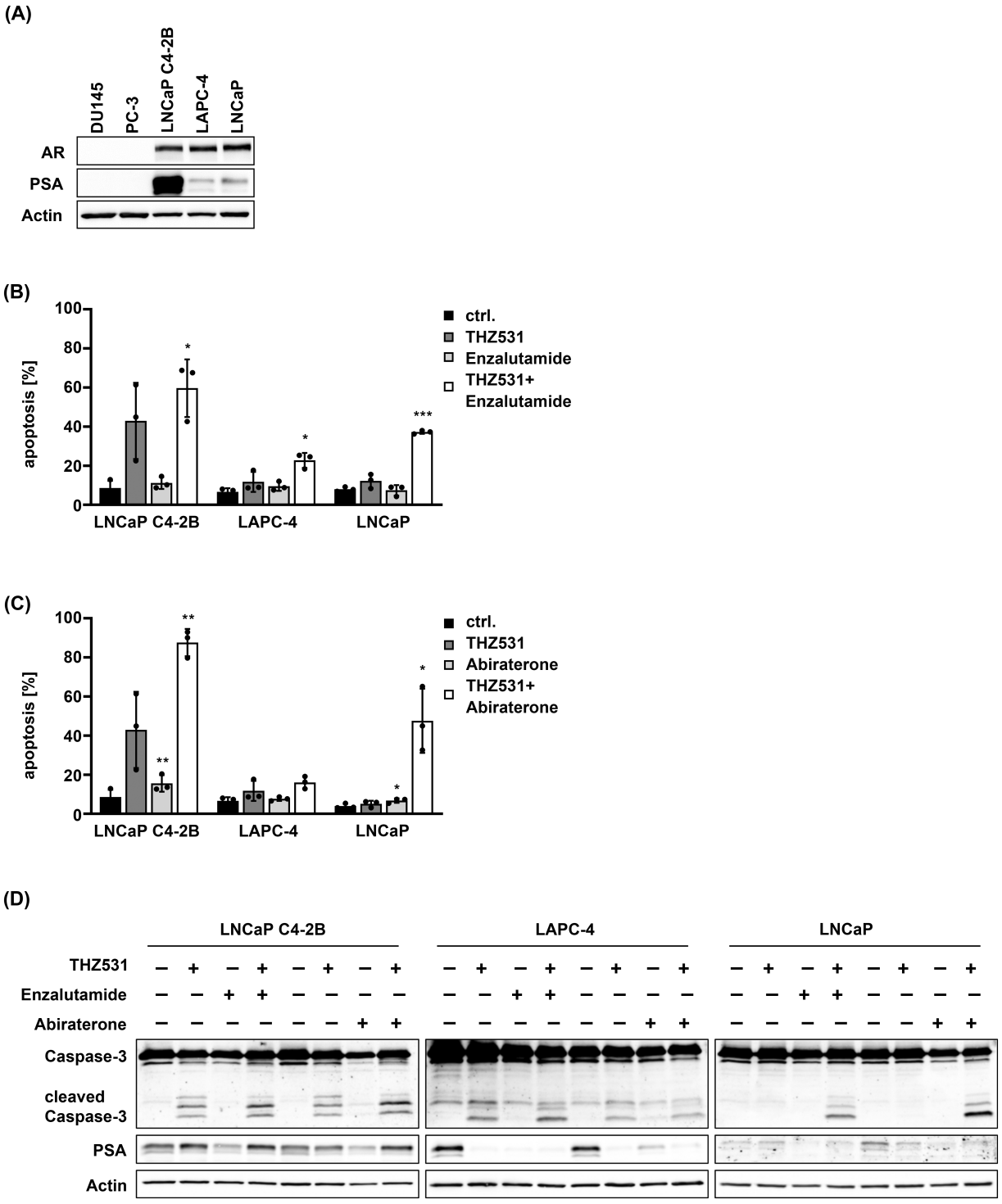

FIGURE 3 The combined inhibition of CycK-CDK12 and AR induces apoptosis in androgen-sensitive PCa cells. (A) Immunoblot analysis of endogenous expression of androgen receptor (AR) and prostate specific antigen (PSA) in several human PCa cell lines. (B-D) PCa cells were treated with 100 nM of THZ531, 10 μM enzalutamide, 10 μM abiraterone or a combination thereof. (B, C) After 72 h of treatment (combination with enzalutamide [B] or abiraterone [C]) LNCaP C4-2B, LAPC-4 and LNCaP cells were stained with 7-AAD and Annexin V and apoptosis rate was detected with subsequent FACS analysis. Data represent the mean of three independent experiments (n = 3 ± SD, paired two-tailed *t* test, $^{*}P < .05$, $^{**}P < .01$). (D) Immunoblot analysis for full-length caspase-3 (p32), and cleaved caspase-3 (p19/p17), and PSA was performed 72 h after treatment.

(Figure 4A-D) or abiraterone (Figure S6A-D) showed significantly reduced mRNA and protein expression levels compared to treatment with THZ531 alone in the androgen-sensitive PCa cell lines. Additionally, an increased amount of γH2AX foci in the nuclei of androgen-sensitive cells after the combined treatment could be detected (Figures 4E, S6E and S7-S9).

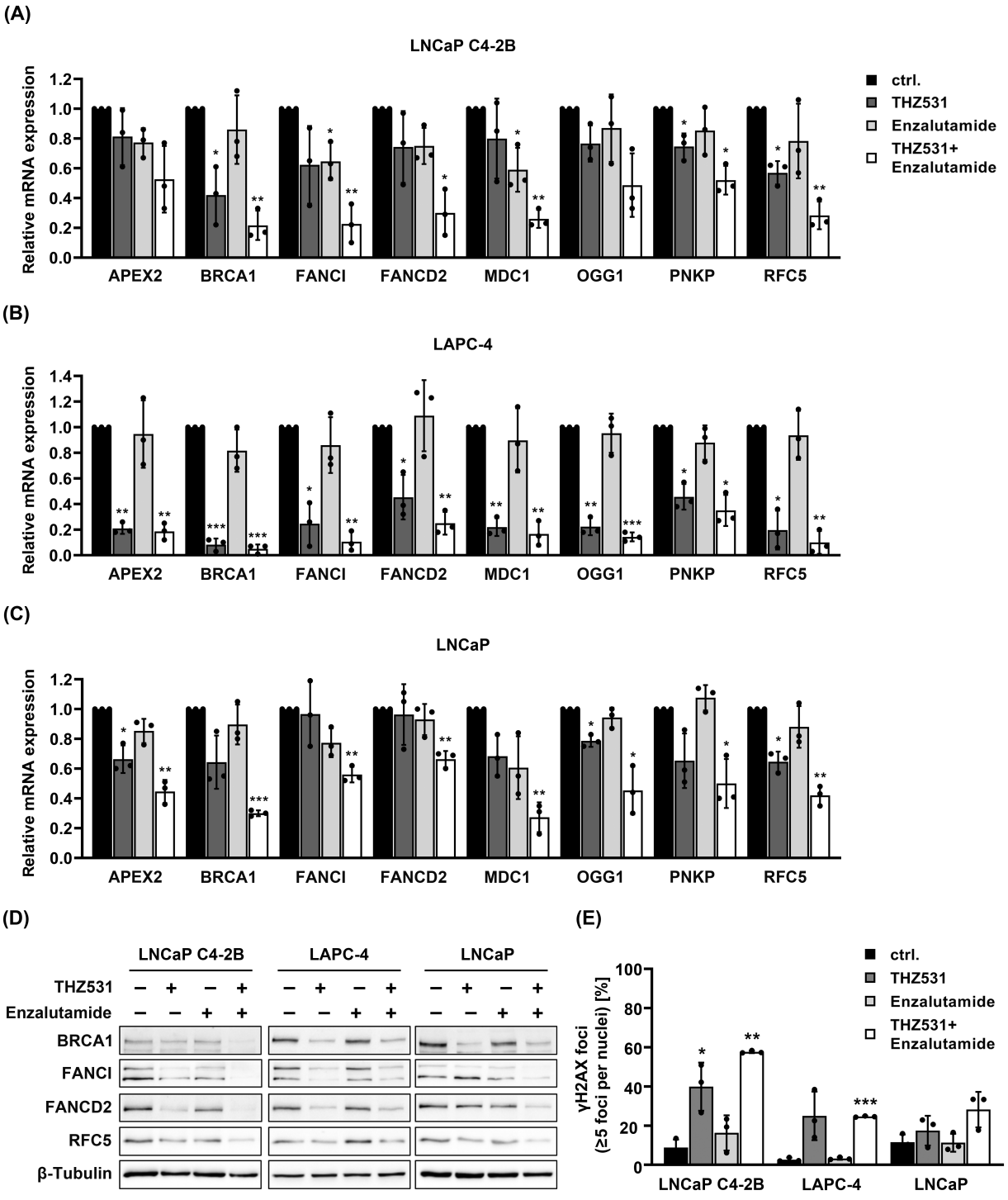

FIGURE 4 The combined treatment of THZ531 and enzalutamide impairs the expression of DNA repair genes and causes DNA Damage in PCa cells. (A-E) PCa cells were treated with 100 nM of THZ531, 10 μM enzalutamide, or a combination thereof. (A-C) RNA was isolated 24 h after treatment and mRNA levels of selected DNA repair genes were assessed by qRT-PCR and normalized to internal 18S rRNA levels. Data represent the mean of three independent experiments (n = 3 ± SD, paired two-tailed *t* test, **P* < .05, ***P* < .01, ****P* < .001). (D) 72 h after treatment, whole cell lysates were prepared. Immunoblot analysis of selected proteins involved in DNA repair was performed. (E) Immunofluorescent staining of PCa cells with an anti-γH2AX antibody with subsequent analysis was determined 72 h post treatment. The percentage of ≥5 γH2AX foci per nucleus were quantified. Data represent the mean of three independent experiments (n = 3 ± SD, paired two-tailed *t* test, **P* < .05, ***P* < .01, ****P* < .001).

3.4 | Simultaneous inhibition of the CycK-CDK12 complex and AR signaling influences proliferation and causes apoptosis in an ex vivo tissue slice model

The investigation of a new therapeutic approach to PCa treatment in a cell culture model is limited due to, for example, a lack of 3D tumor structure, tumor heterogeneity, and tumor microenvironment. Therefore, we used a more appropriate method—an ex vivo tissue slice model, which has been established in our lab.[21] PCa tumor tissue from patients after radical prostatectomy was cut into 300 µm thick slices and treated with THZ531, enzalutamide (n = 12), or abiraterone (n = 6), or a combination thereof. In addition, the expression of CycK, CDK12, and a functional AR was confirmed by immunohistochemistry in untreated and non-cultured prostate tissue from each patient (Figure S10A). The clinicopathological features of these patients are summarized in Table S3. To determine the consequences of CycK-CDK12 and AR inhibition on response to therapy, an immunohistochemical staining of Ki67 (proliferation) and cleaved caspase-3 (apoptosis) was performed with subsequent QuPath analysis. Representative pictures of the immunohistochemical staining are shown in Figure S10B-C. The individual examination of each sample showed a broad spectrum from therapy resistance to a high therapy response in the PCa patient collective (Figures S11-S13). Overall, a significant decrease of Ki67-positive tumor cells was observed after treatment with THZ531 and enzalutamide compared to THZ531 alone (Figure 5A). Similarly, combination treatment with abiraterone reduced Ki67-positivity, although not to a significant extent (Figure 5B). Furthermore, treatment with THZ531 in combination with enzalutamide increased the number of apoptotic tumor cells (Figure 5A). A higher significant number of cleaved caspase-3-positive tumor cells was observed with THZ531 alone and the combination with abiraterone as well (Figure 5B).

3.5 | Cyclin K acts as a prognostic biomarker for PCa

Previous studies reported that the CycK-CDK12 complex is a promising target for cancer therapy, a predictive biomarker for patients with PCa, and is suspected to be a prognostic marker in cancers.[13,14] To determine the eligibility of CycK and CDK12 as prognostic biomarkers, expression of both proteins was immunohistochemically analyzed in PCa tissue using a TMA. The TMA collective includes 300 patients with PCa who received no neoadjuvant therapy. Table S4 summarizes the clinicopathological features of this patient cohort. Immunohistochemistry showed a heterogeneous nuclear and/or cytoplasmic CycK and CDK12 expression in prostate tumor tissue, which was graded according to intensity in low, medium, and high (Figures 6A and S14A). Correlation analysis of CycK expression with clinicopathological features revealed that increasing CycK expression levels were significantly associated with the grading group ($P \leq .001$), tumor extent ($P \leq .05$), lymph node metastasis ($P \leq .01$), and perineural invasion ($P \leq .001$; Figure 6B). In contrast, no association between CDK12 expression and clinicopathological features was detected with exception of Pn1-positive tumors that showed a lower expression (Figure S14B). Similar observations could be made after dichotomizing the TMA collective according to CycK and CDK12 expression (50% high, 50% low; Table S5). Here, however, no significant association between CycK expression and tumor extent could be demonstrated.

4 | DISCUSSION

Progression of PCa to metastatic state is characterized by inevitable evolvement of treatment resistance. One of the cornerstones of the first-line treatment for advanced PCa is inhibition of AR signaling. However, in many cases therapy fails, so that new targeted therapeutics are urgently needed. The recent discovery of the covalent CDK12 inhibitor THZ531 now enables to investigate the effects of targeted inhibition of the CycK-CDK12 complex.[10] In the current study, we demonstrated the suitability of CDK12 and its regulatory subunit CycK as a novel therapeutic target and prognostic biomarker in PCa.

First, we determined the on-target activity of THZ531 based on CycK-CDK12-mediated phosphorylation of Ser2, Ser5 or Ser7 residues in the CTD of RNAP II. Consistent with the literature, we confirmed that THZ531 dose-dependently suppressed the phosphorylation of the serine residues in the CTD of the RNAP II, especially Ser2, in PCa cells.[25] Functionally, inhibition of the CycK-CDK12 complex induced either apoptosis or G2/M phase arrest of the cell cycle in PCa cells in a cell type dependent manner. Furthermore, in an avian model, THZ531 treatment resulted in decreased tumor viability, which was accompanied by elevated rates of apoptosis. This result is in agreement with other studies reporting that depletion of CycK using siRNA induces apoptosis in DU145 cells[13] and that THZ531 induces apoptosis in leukemia cells.[10] In addition, Blazek et al. showed that siRNA-mediated reduction of either CycK or CDK12 in HeLa cells resulted in an accumulation of cells in the G2/M phase.[8] Besides inducing a G2/M phase arrest, inhibition of the CycK-CDK12 complex might also result in delayed G1/S phase progression.[9] Similarly, the knockdown of CycK led to G1/S cell cycle arrest in colon carcinoma cells, which is attributed to the prevention of the formation of the prereplicative complex by CycK-CDK12-dependent phosphorylation of Cyclin E1.[12] Furthermore, CDK12-mediated RNAP II processivity has been described as a rate-limited factor for optimal S phase entry and cellular proliferation.[9] The observed cell cycle arrest and apoptosis induction are most likely a consequence of the THZ531-mediated induction of DNA damage through reduced expression of DDR genes, which we and others have demonstrated.[8] Consistent with our data, it has already been shown that CycK-CDK12-regulated transcription is essential for the expression of HR genes, including BRCA1, ATM, and Fanconi anemia genes.[8,10,15] This can then result in inefficient HR repair leading to replication stress, followed by genome instability for example, in the form of tandem duplications,[9] which are already observed in tumors with mutated CDK12.[17,18,27] In addition, a previous study showed a CDK12-dependent translation of mRNAs

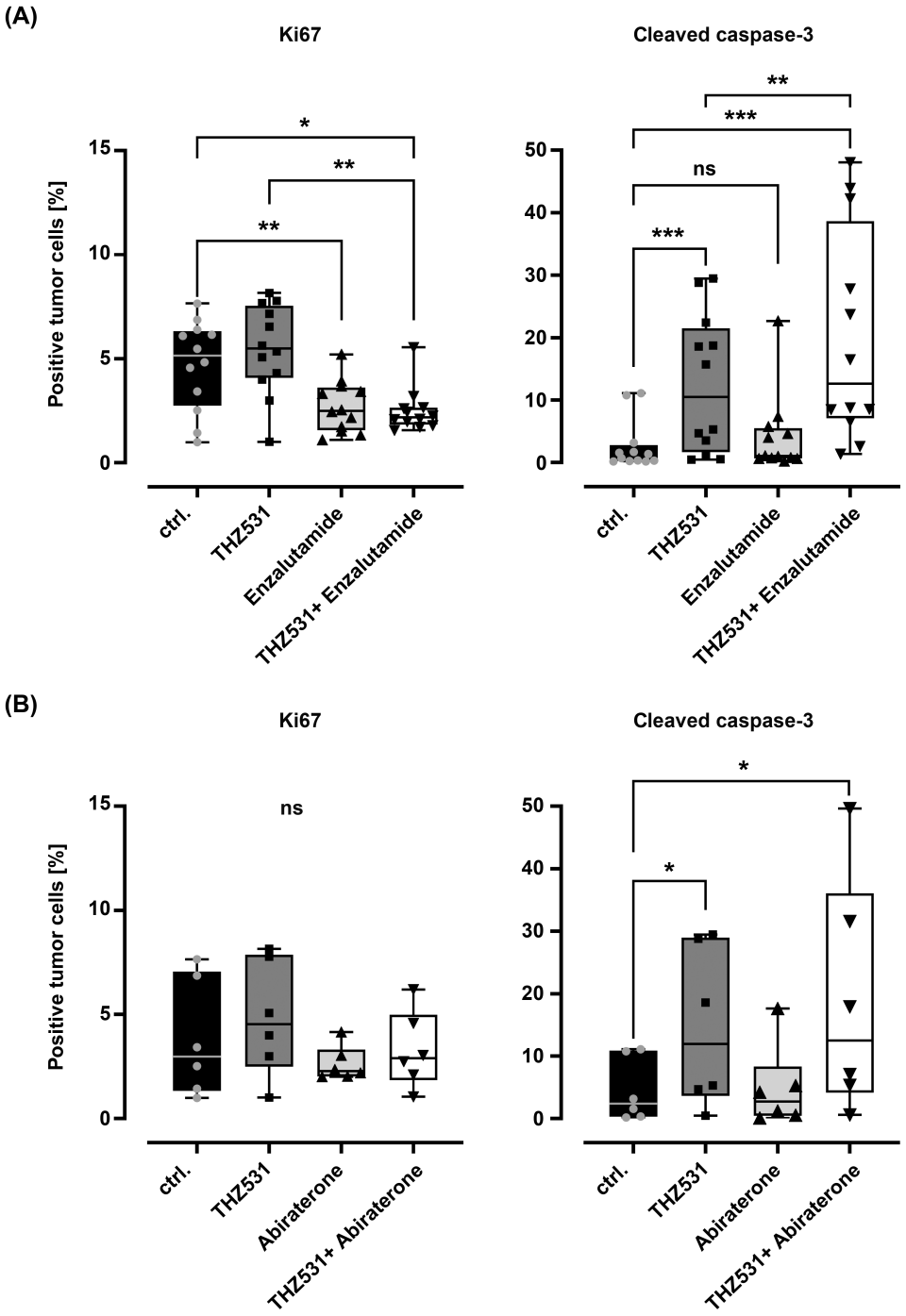

FIGURE 5 The combined inhibition of CycK-CDK12 and AR influences proliferation and causes apoptosis ex vivo. PCa tissue slices were cultivated ex vivo and treated with 500 nM THZ531, 10 μM enzalutamide (A), 10 μM abiraterone (B), or a combination thereof for 72 h. Immunohistochemical staining of cleaved caspase-3 and Ki67 were conducted and analyzed by digital image analysis. (A) Data represent the quantification of the fraction of KI67-positive tumor cells and cleaved caspase-3-positive tumor cells of 12 patients. (B) Data represent the quantification of the fraction of KI67-positive tumor cells and cleaved caspase-3-positive tumor cells of six patients. Each point represents the mean of three tumor slices per treatment from every patient (boxplots show mean ± SD, paired two-tailed *t* test or Wilcoxon signed-rank test, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, ns, non-significant).

encoding subunits of mitotic and centromere complexes as well as chromosomal misalignment upon CDK12 and CycK depletion.[28] Similarly, downregulation of CDK12 or CycK resulted in mitotic defects such as an Aurora B kinase-dependent mitotic catastrophe in PCa cells,[13] which led to apoptosis. Of note, THZ531 selectively targets both CDK12 and its homolog CDK13.[10] Therefore, reduced expression of DNA repair genes could be attributed to both kinase activities. However, several studies have shown that CDK12 predominantly regulates the expression of DDR genes, whereas CDK13-dependent genes are more involved in posttranscriptional modification of rRNA or various extracellular and growth signaling pathways.[11,25] From this, we conclude that the CycK-CDK12 complex alone is responsible for the transcriptional regulation of DDR genes.

In the present study, we observed different effects of CycK-CDK12 inhibition in a cell type depending manner: apoptosis induction in DU145 and LNCaP C4-2B cells, or inhibition of cellular proliferation in G2/M phase in PC-3 and LAPC-4 cells, respectively. Upon DNA damage, cell cycle checkpoints are activated and the cell cycle is arrested to allow the DNA repair machinery to restore genomic integrity. However, when DNA repair fails and the integrity of the genome is irreparably compromised, the cell undergoes apoptosis.[29]

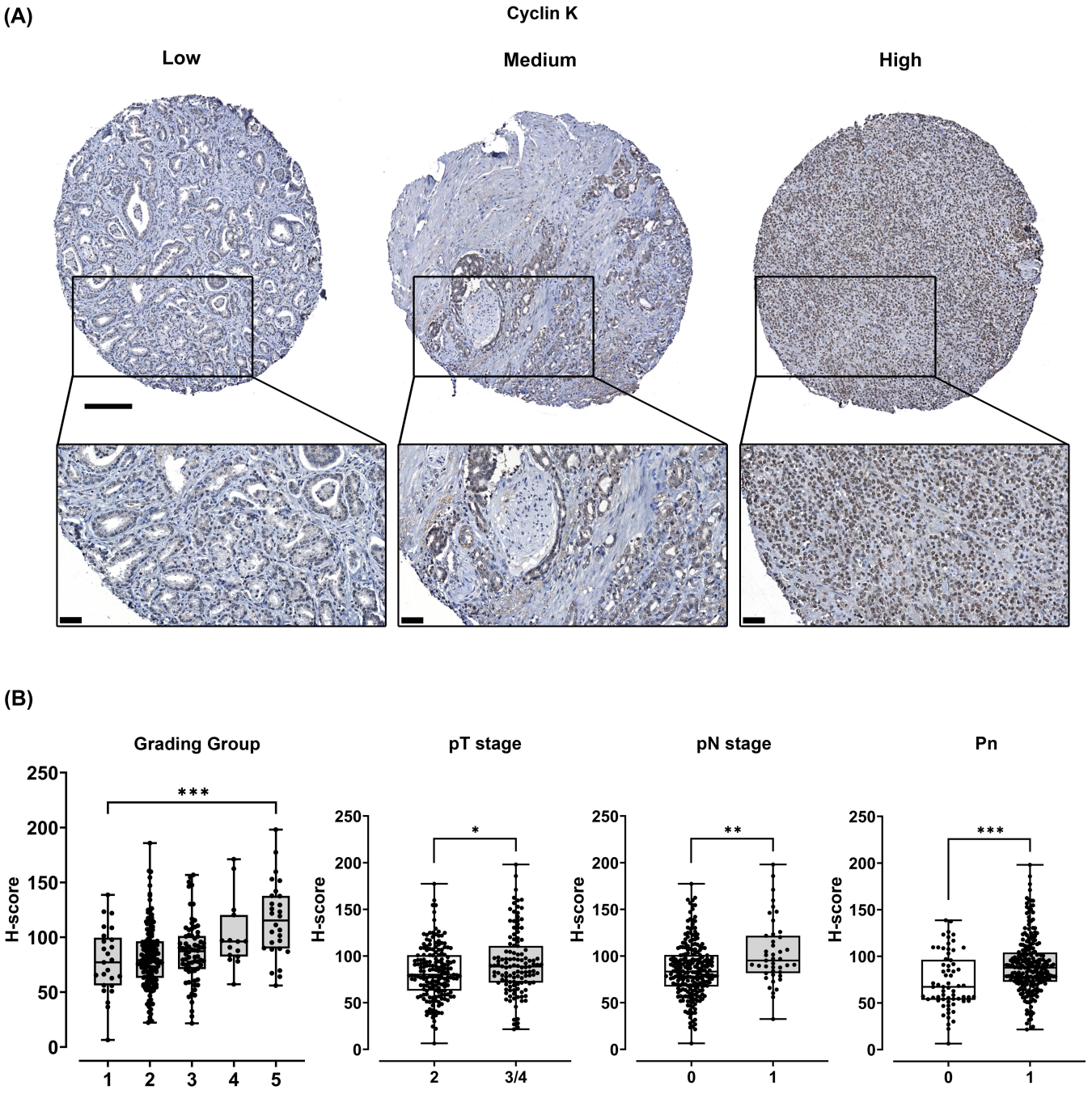

FIGURE 6 An increased CycK expression is associated with a more aggressive tumor phenotype. (A) Overview (upper panel) and higher magnification (lower panel) of immunohistochemical staining representing a low, medium, and high intensity of CycK expression of PCa tissue core from the TMA collective (upper panel: scale bar = 200 µm, lower panel: scale bar = 50 µm). (B) Comparison of CycK expression with clinicopathological features. Expression levels of CycK were calculated by H-score for each prostate tumor core using QuPath software and graphed as boxplots. Each point represents the mean of four tumor cores from each patient (n = 300 ± SD, Kruskal-Wallis test ***P < .001; Mann-Whitney U test *P < .05, **P < .01, ***P < .001).

Comparison of PC-3 and DU145 cells using the Cancer Dependency Map data set (https://depmap.org/portal/) showed that PC-3 cells harbor no mutations in essential DDR genes such as BRCA1/2, RAD50 and XRCC1. In contrast, mutations in these and other important DDR genes were found in DU145 cells. The observed responses to THZ531 could therefore be attributed to the different efficiencies of the DNA repair machinery. Strikingly, in contrast to its derivative LNCaP C4-2B, no pronounced effect of THZ531 on LNCaP cells was detected, although THZ531 suppressed the phosphorylation of the CTD of RNAP II at Ser2. Both cell lines differ in a variety of mutations and different expression profiles.[20] For example, the expression of the p21-activated kinase PAK1 is significantly reduced in LNCaP C4-2B

cells.[20] Since a decreased expression of PAK1 could lead to a reduced expression of Fanconi anemia family genes resulting in an increased sensitivity to DNA-damaging agents,[30] its elevated expression in LNCaP cells may counteract inhibition of the CycK-CDK12 complex. Moreover, PAK1 is able to stimulate the transcriptional activity of the AR,[31] which also positively regulates the expression of DDR genes.[26,32] However, the role of PAK1 in susceptibility to THZ531 remains to be investigated. Besides, the tumor suppressor genes TP53 and PTEN are critical contributors to cell fate by promoting cell cycle arrest, DNA repair, and cell death.[33] However, in the cell lines DU145 ($p53^{mut}$, $PTEN^{wt}$), PC-3 ($p53^{del}$, $PTEN^{del}$), LNCaP ($p53^{wt}$, $PTEN^{mut/del}$), LNCaP C4-2B ($p53^{wt}$, $PTEN^{mut/del}$) and LAPC-4 ($p53^{mut}$, $PTEN^{wt}$), no apparent correlation between the mutation status of these genes and the response to THZ531 could be observed, so other factors seem to play a role in this context.[19,34] In addition, we found that simultaneous treatment with THZ531 and either enzalutamide or abiraterone caused apoptosis in LNCaP cells and increased the rate of THZ531-induced apoptosis in LNCaP C4-2B and LAPC-4 cells. This finding is consistent with a recent study detecting more apoptotic cells after combining enzalutamide and THZ531 in PCa cells.[16] These elevated apoptosis rates are most likely due to the role of the AR signaling pathway in the transcriptional regulation of DDR genes.[26,32] Accumulating evidence revealed, that the AR transcriptionally upregulates several DDR genes in PCa.[26,32] Accordingly, the combination of THZ531 and ADT led to a further reduction in the expression of DDR genes and consequently to a further increase in DNA damage. The link between AR signaling and transcription of DDR genes is of particular importance, as this property can be exploited for therapeutic combinations to induce synthetic lethality.[35]

In the present study, we show for the first time that combined inhibition of the CycK-CDK12 complex and AR signaling reduces the number of proliferating cells and causes apoptosis ex vivo in PCa tissue, thereby confirming the results of our in vitro experiments. The tissue slice culture model offers several advantages over traditional cell culture. Since the tissue samples preserve their original tumor stroma and a three-dimensional architecture, the comparability of the ex vivo effects with the in vivo situation is increased.[36] In addition, the treatment of fresh patient-derived tumor tissue gives us the opportunity to study the response of the tumor to the inhibitors directly. Thus, the high intra- and intertumoral heterogeneity can be better taken into account. In our study, we observed a wide range from therapy sensitivity to resistance, which can be attributed to the high tumor heterogeneity. However, further study is required to gain more insight into the underlying mechanism that is responsible for the different treatment responses. Within this context, mutations in the CDK12 gene, which occur in approx. 7% of PCa might be associated with a poor response to THZ531.[37] In addition, the impact of other PCa-associated mutations, especially TMPRSS2 and PTEN, which are both involved in DNA damage response,[33,38] still needs to be investigated.

In contrast to CDK12, little is known about the potential of CycK as a possible biomarker in PCa. To date, CycK expression has been studied in testicular tumors, lung cancer and as well in breast cancer patients. In all cases, an increased CycK expression was found in tumor tissue compared to the respective normal tissue.[12,39,40] In addition, we recently investigated the expression of CycK in PCa tumors with regard to its suitability as a prognostic and predictive biomarker.[13] However, the patient cohort used in our previous study had some disadvantages such as the small number of tumor samples ($n = 91$) and the inclusion of only high-grade tumors with a Gleason score ≥ 7. In order to improve the power of conclusion of this study, we generated a TMA that included both an increased number of patient samples ($n = 300$) and a balanced ratio of low-grade and high-grade tumors. We found that high CycK expression levels were significantly associated with a more aggressive tumor phenotype. Moreover, analysis of the publicly available survival data from the Cancer Genome Atlas through the platform cBioPortal[41] revealed that increased CycK mRNA expression levels were significantly correlated with a shorter progression-free survival (data not shown). This could be attributed to the pro-proliferative and anti-apoptotic role of CycK in PCa. In addition, this correlation could also be due to the newly identified role of CycK as a regulator of Wnt/β-catenin signaling.[40] CycK interacts with β-catenin, the transcriptional co-regulator of Wnt target genes, thereby upregulating the expression of Cyclin D1 promoting tumorigenesis in lung cancer.[40] Interestingly, previous studies have found increased Cyclin D1 expression in high-grade prostate tumors[42] as well as an increased accumulation of nuclear β-catenin in prostate tumor specimens with high Gleason scores.[43] Further, there is evidence that the Wnt/β-catenin pathway is active in advanced stage PCa and its activation enables castration-resistant growth and induction of epithelial-to-mesenchymal transition.[43,44] Therefore, it might be possible that increased expression of CycK enhances Wnt/β-catenin signaling and thereby contributes to tumor development and progression.

Surprisingly, no association was found between CDK12 expression and other clinicopathological features with exception of Pn1-positive tumors in our patient collective. In contrast, higher CDK12 expression in gastric tumor tissue has been found to be associated with malignant phenotypes and poor outcomes in these patients.[45] This different prognostic importance of CycK and CDK12 suggests that both proteins may have different relevance in PCa progression. Of note, CycK not only interacts with CDK12 but is also a binding partner of CDK13.[7,10] It was recently shown that the genes regulated by CDK12 or CDK13 overlap by about 75%, which indicates a partial functional redundancy of the two kinases.[11] It is therefore possible that no prognostic statement can be made by examining the expression of CDK12 in PCa alone and that the expression of CDK13 should also be taken into account.

Our findings were consistent with several studies highlighting the role of the CycK-CDK12 complex in tumorigenesis and the potential of CycK-CDK12-targeting therapies in various tumor entities. CDK12 has been shown to be an important tumor suppressor involved in maintaining genome stability.[10,12,25] Mutations, deletions, or a lack of CDK12 expression are commonly found in breast cancer, ovarian carcinoma, and PCa showing HR deficiency characterized by reduced expression of DDR-related genes.[14,15,17,46] These tumors are sensitive

to DNA-damaging agents and PARP inhibitors. Consistently, our data showed that inhibition of CycK-CDK12 increased sensitivity to the PARP1 inhibitor olaparib in DU145 and LNCaP C4-2B cells and particularly in LNCaP cells.

In addition, promising clinical trials using PARP inhibitors alone[47] or in combination with enzalutamide[48] or abiraterone[49] taking advantage of synthetic lethality are currently ongoing, especially in mCRPC harboring HR defects. Furthermore, PCas with CDK12-associated focal tandem duplications can lead to gene fusion, subsequently producing fusion-induced neoantigens, which increases sensitivity to immune checkpoint inhibitors.[17,18] In this context, we and others have shown that the newly synthesized CDK12/13 inhibitor THZ531 may represent an effective therapeutic strategy: THZ531 has been reported to synergize with PARP inhibitors in Ewing sarcoma[50] and increases the sensitivity of anaplastic thyroid carcinoma cells to chemotherapy.[51] Similarly, THZ531 suppresses the expression of DDR genes, induces a potent DDR and acts synergistically with sorafenib in hepatocellular carcinoma.[24] In addition, the present study demonstrated a THZ531-induced susceptibility of both PCa cells and tissue to ADT.

In summary, our study suggests a clinical relevance of the CycK-CDK12 complex as a promising target for combination therapy with ADT in PCa. The combination of THZ531 and ADT led to a reduced expression of DDR genes, which was accompanied by the induction of DNA damage and apoptosis, and thus offers first indications of the anti-tumorigenic potential of this combination therapy both in vitro and ex vivo. Moreover, we identify CycK as a prognostic biomarker for patients with PCa.

AUTHOR CONTRIBUTIONS

The study reported in the article has been performed by the authors, unless clearly specified in the text. Katrin E. Tagscherer, Wilfried Roth: conceptualization and design. Katharina Frei, Sabrina Schecher, Ute Hildebrand, Jutta Richter, Nina Hörner: acquisition of data. Katharina Frei, Sabrina Schecher, Katrin E. Tagscherer, Wilfried Roth, Stefan Porubsky, Hagen R. Witzel, Matthias M. Gaida: analysis and interpretation of data. Tamas Daher: pathological data. Igor Tsaur: collection of clinical data. Mario Schindeldecker: statistical analyses. Katharina Frei, Katrin E. Tagscherer: drafting of the manuscript. Wilfried Roth, Katrin E. Tagscherer: project administration and supervision. All authors were involved in writing or revising the article and had final approval of the submitted and published versions.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are part of the main article including figures, tables, and supplementary information. The tissue investigated for this study is archived in the Institute of Pathology of the University Medical Center Mainz. The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The study was performed with the approval of the Tissue Biobank of the University Medical Center Mainz (ref. no. 837.359.17 [11193]) and in accordance with the regulations of the Tissue Biobank and the ethics vote of the medical association of the State of Rhineland-Palatinate (ref no. 837.360.16 [10679]). All experiments performed on fertilized eggs prior to hatching do not require the approval of the Animal Welfare Act (TierSchG) and the Animal Welfare Laboratory Animal Regulations (TierSchVerV).

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SUPPORTING INFORMATION

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

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