



# Protein kinase D activity is a risk biomarker in prostate cancer that drives cell invasion by a Snail/ERK dependent mechanism

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## ABSTRACT

Protein kinase D (PKD) family members play controversial roles in prostate cancer (PC). Thus, PKD1 is nearly absent in advanced tumours, where PKD2 and PKD3 are upregulated. Additionally, consequences of activation of these kinases on PC progression remain largely unclear. Here, we first investigated PKD function on PC cell motility, analysing the underlying molecular mechanisms. We find a striking decrease of Snail levels after PKD inhibition followed by cell migration and invasion impairment, demonstrating an unprecedented role of PKD activity on the regulation of this key transcription factor in PC progression. Specifically, we show that PKD2 activity mediates the effects of MEK/ERK pathway on Snail expression, establishing a joint function of ERK/PKD2/Snail cascade in PC cell invasion regulation. These results led us to address the clinical relevance of the correlation between PKD2 and ERK activities with Snail abundance in samples from PC patients at different stages, analysing its impact on tumour prognosis and patients' survival. Importantly, this is the first study defining a direct correlation between active PKD2 and Snail levels, further linked to ERK activity. We also evidence that PKD2 activity is associated with important poor prognostic factors. Thus, PC patients with the expression pattern: active PKD2<sup>high</sup>/active ERK<sup>high</sup>/Snail<sup>high</sup> exhibit increased invasiveness and metastasis, and decreased survival. Our findings provide new insights for understanding the molecular mechanisms involved in PC progression, pinpointing the combination of active PKD2 and Snail levels, with the additional measurement of active ERK, as a confident biomarker to predict clinical outcome of patients with advanced PC.

## 1. Introduction

Prostate cancer (PC) is a global health problem, being the second most common cancer and the fifth cause of cancer death in men [1]. Mortality is mainly due to metastasis, which is associated with the ability of tumour cells to increase their migration and invasion rates [2]. We have previously described that MAPK signalling cascades and the dual specificity phosphatase 1 (DUSP1), as well as the increased expression of the transcription factor Snail are important mechanisms

involved in PC progression [3–5]. We also evidenced in samples from patients with PC that correlation between expression and/or activation levels of these molecules is an important prognostic factor to classify PC stages, thus demonstrating the clinical relevance of our previous findings [3,5].

Protein kinase D (PKD) family of kinases is constituted by three isoforms (PKD1, PKD2 and PKD3), of which both PKD1 and PKD2, but not PKD3, when active are autophosphorylated at residue Ser<sup>910</sup> and Ser<sup>876</sup>, respectively [6]. These proteins exert essential pro-survival roles

**Abbreviations:** BPH, Benign prostatic hyperplasia; CRT, CRT0066101; DUSP1, Dual specificity phosphatase 1; EMT, Epithelial-to-mesenchymal transition; GSK-3 $\beta$ , Glycogen synthase kinase-3 $\beta$ ; HR-PC, Hormone refractory-PC; HS-PC, Hormone responsive-PC; PC, Prostate cancer; PKD, Protein kinase D; PSA, Prostate-specific antigen; TCP, Time to clinical progression.

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in different physio-pathological situations [6,7], however, their contribution to cancer is very controversial depending on the isoform and the tumour type [8,9]. Specifically in PC, the studies analysing the role of PKD expression are contradictory. Thus, PKD1 expression is mainly downregulated or even absent in the most aggressive tumours [10–13], as well as in androgen-independent cell lines [14,15], and the expression of this isoform is broadly associated to an inhibition of PC cell proliferation and migration [8–11,16]. By contrast, PKD2 and PKD3 are highly expressed in advanced PC human samples and androgen-independent cell models [14–18], promoting cell proliferation, survival, migration and invasion [11,14,17]. Despite the opposite roles of the expression of different PKD isoforms, several reports using diverse pan-PKD inhibitors evidence impaired proliferation, migration, and invasion of PC cells. However, most of these compounds show lack of selectivity, targeting a few additional kinases, and their mechanisms of action have not been analysed in detail [19–23]; hence the role of PKD activity in PC progression is an important question that remains unclear. In this study we have addressed the molecular mechanisms implicated in PC progression that are affected by PKD activity by employing the CRT0066101 (CRT) molecule, which is the most selective and highly effective pan-PKD inhibitor to date, rendering it as an ideal candidate for clinical development [23]. In addition, we have used an antibody recognizing Ser<sup>910</sup> and Ser<sup>876</sup> that allows to selectively determine the activity of PKD1 and PKD2, therefore we can attribute the signal detected by this antibody to PKD2 activity in our cell lines in particular, in which PKD1 expression is negligible [14,15].

Here, we have first investigated the effect of PKD inhibition on PC tumour cell progression, finding significant decreases in cell viability, migration and invasion together with downregulation of Snail levels. We have also discovered the activation of glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), a main regulator of this transcription factor, and the striking proteasome-dependent degradation of Snail upon CRT treatment, that is accompanied by the nuclear exit of this transcription factor. It is noteworthy that, considering that PKD1 expression is almost undetectable in our cell models [14,15], the other two PKD isoforms must be responsible for the majority of the effects observed in response to the CRT inhibitor. Furthermore, here we show that Snail protein levels highly correlate to PKD2 activity, which in turn mediates the effects of ERK signalling pathway on the expression of this transcription factor. Of important clinical relevance, in this study we observe high expression of active PKD2 (pPKD2), active ERK (pERK) and Snail, with a parallel decrease in DUSP1 levels, in samples from patients with advanced PC. A key point in our study is that high pPKD2 levels are significantly associated with most PC poor prognostic factors, including hormone resistance, Gleason score, clinical progression, invasiveness, metastasis, and exitus rate. All these findings are extraordinarily important since they suggest that the use of the expression pattern pPKD2<sub>high</sub>/pERK<sub>high</sub>/Snail<sub>high</sub> might be a novel diagnostic marker in advanced prostatic tumours, opening the possibility to improve current therapeutic strategies.

## 2. Materials and methods

### 2.1. Cell lines, inhibitors, plasmids, siRNAs oligos, cell transfection, and luciferase assay

DU145 and PC3 cells were purchased from the American Tissue Culture Collection (Manassas, VA, USA) and were cultured as recommended. The inhibitors were CRT0066101 (Abcam, Cambridge, UK), U0126 (Promega Biotech Ibérica, Madrid, Spain), SB203580, SP600125 and MG132 (Calbiochem, Merck Chemicals, Barcelona, Spain). The Snail-Luc reporter plasmid was previously described [24], and luciferase assays were performed as in previous studies [25]. GFP control plasmid was EGFP-C2 (BD Biosciences Clontech, Palo Alto, CA). The constitutively active PKD2 plasmid (GFP-caPKD2), and the constitutively active BRAF plasmid (V<sup>600E</sup>BRAF) were previously described in [26] and in [27], respectively. DUSP1 siGENOME (M-003484-02-0010, smart pool)

and PKD2 siGENOME (M-004197-02, smart pool) were obtained from Dharmacon (Lafayette, CO, USA). Silencer<sup>TM</sup> negative control#1 specific siRNA was obtained from Ambion (Carlsbad, CA, USA). For over-expression and silencing experiments, cells were transiently transfected as previously described [4].

### 2.2. Western blot analyses and immunofluorescence staining assays

Western blot analyses were performed as previously described [25]. The antibody recognizing both phosphorylated Ser<sup>910</sup> in PKD1 and Ser<sup>876</sup> in PKD2, anti-PKD2, anti-phospho-ERK (pERK), anti-phospho-p38MAPK (pp38MAPK), anti-phospho-Ser<sup>9</sup>-GSK-3 $\beta$  (pGSK-3 $\beta$  (S<sup>9</sup>)), and anti-Snail were from Cell Signalling Technology (Werfen S.A., Barcelona, Spain). Anti-ERK2, anti-p38MAPK, anti-JNK1, anti-DUSP1 and anti-BRAF were from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-phospho-JNK (pJNK) was from Promega Biotech Ibérica (Madrid, Spain). Anti-GFP was from Proteintech (Barcelona, Spain). Anti-tubulin was from Sigma Aldrich (Madrid, Spain). Peroxidase-conjugated secondary antibodies were from GE Healthcare Europe GMBH (Barcelona, Spain). Tubulin was utilized as a loading control for Western blotting analysis. Relative protein levels compared to tubulin were analysed by Image J software and plotted. Snail immunofluorescence staining of cultured PC cells was carried out as previously described [28]. Images were acquired using an inverted Zeiss LSM710 laser confocal microscope, processed for presentation with Zen2.3 SP1 and ImageJ, and presented as two-dimensional maximal projections of a z-series.

### 2.3. Cell migration and invasion assays

Cell migration was examined by wound-healing assays, and the percentages of wound closure and migration velocities were quantitated as previously described [5]. Cell invasion was examined in Matrigel-coated transwells (BD Biosciences, Franklin Lakes, NJ) as in previous studies [5,29].

### 2.4. Experimental subjects and immunohistochemistry of prostate tissues

The cohort of paraffin-embedded samples from patients diagnosed with prostate adenocarcinoma ( $n = 35$ ) were evaluated (Table 1). Benign prostatic hyperplasia (BPH) samples obtained from simple

**Table 1**  
Clinical characteristics of PC patients ( $n = 35$ ).

	n (%)
Age	
<70 years	22 (62.9)
≥70 years	13 (37.1)
Total Gleason score	
6	1 (2.9)
7	12 (34.3)
8	13 (37.1)
9	7 (20.0)
10	2 (5.7)
T category (Invasiveness)	
T1	5 (14.3)
T2	11 (31.4)
T3	15 (42.9)
T4	4 (11.4)
Metastatic disease	
Non metastatic (N0, M0)	29 (82.9)
Metastatic (N1–2, M1)	6 (17.1)
Hormone refractory-PC (HR-PC)	
No	20 (57.2)
Yes	15 (42.8)
Treatment/surgery	
Radical prostatectomy	16 (45.7)
TUR-P* + medical castration	19 (54.3)

\* Transurethral resection of the prostate.

adenectomy in patients without clinicopathological evidence of PC ( $n = 9$ ) were also evaluated as controls. Patients with clinically localized disease (T1-T2) were treated with radical prostatectomy, while patients with locally advanced disease or pelvic mass (T3-T4) and patients with overt metastatic disease (M1) received transurethral resection of the prostate (TUR-P) and medical castration using luteinizing hormone-releasing hormone analogues. Hormone refractory-PC (HR-PC) was defined as disease relapse despite appropriate castration (serum testosterone  $<50$  mg/mL) and confirmed biochemical progression with over 25 % increase in Prostate-specific antigen (PSA) within two consecutive measurements separated by at least one week and an absolute value above 2.0 ng/mL. Clinical evolution was based on PSA levels and imaging analysis. Primary endpoint assessed was time to clinical progression (TCP), defined as the appearance of two or more bone lesions on bone scan or enlargement of a soft tissue lesion using RECIST [30]. Immunohistochemistry assays were performed as in previous studies [5]. The primary antibodies were the same used for Western blot analysis, with the exception of anti-Snail, clone G7 from Santa Cruz Biotechnology. Immunostaining intensity was evaluated by two independent observers, and immunostaining scores were ranged into four categories as previously described [5].

## 2.5. Statistical analyses

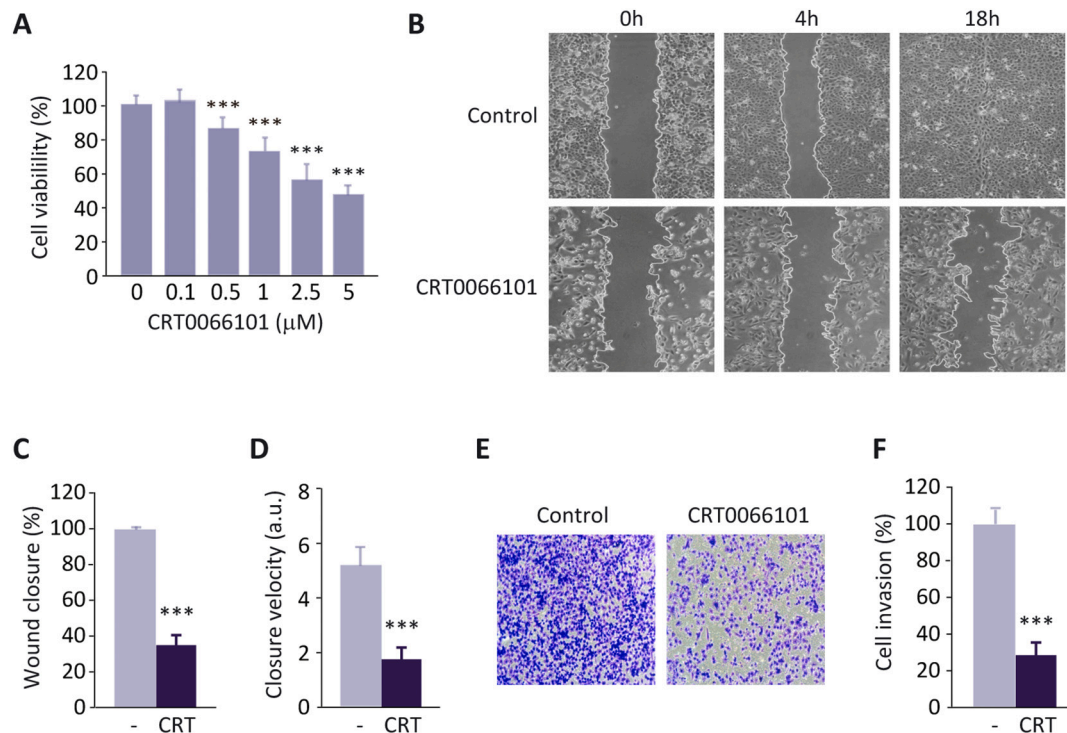
In the experiments with cell lines, data were expressed as means  $\pm$  SEM. Student's *t*-test was performed using the SSC-Stat software (V2.18, University of Reading, UK). In the immunohistochemistry analyses, GraphPad Prism 3.0 software was used for statistical purposes. Immunostaining score and clinical data were analysed using one-way ANOVA and either the Bonferroni's or Dunnett's multiple comparison tests. The correlation among markers was analysed using the Pearson's test (95 % confidence interval). Log-rank test and survival curves were used to determine the relationship among markers and time to clinical

progression (TCP). The statistical significance of difference between groups was expressed by asterisks (\*  $0.01 < p < 0.05$ ; \*\*  $0.001 < p < 0.01$ ; \*\*\*  $p < 0.001$ ).

## 3. Results

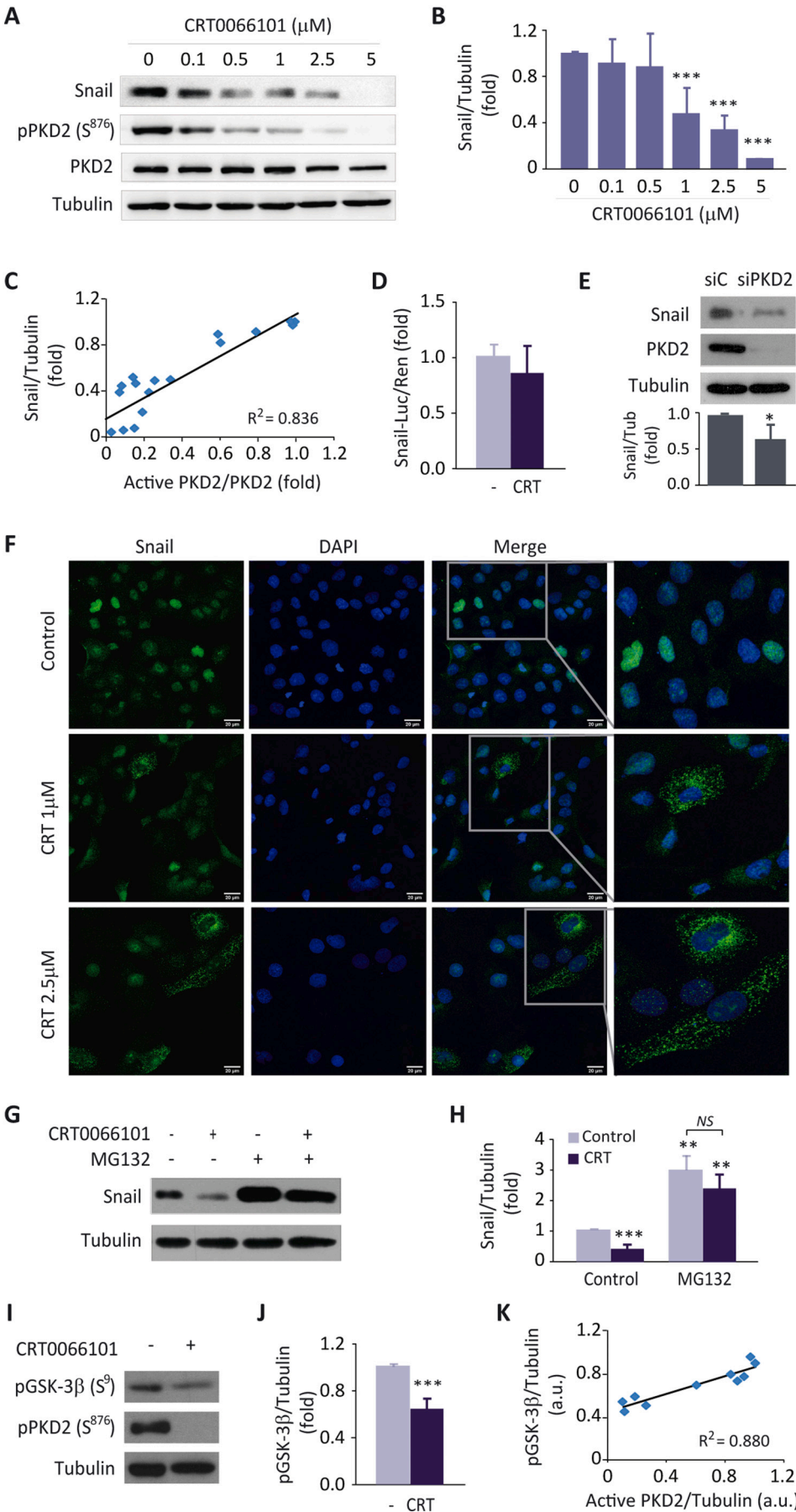
### 3.1. PKD inactivation impairs cell viability, migration and invasion in PC cells, and decreases Snail levels, inducing its nuclear exit and proteasomal degradation

In order to clarify the role of PKD activity in PC, we tested the effects of its inhibition with the CRT compound in the PKD1-non expressing DU145 cell line [14,15]. First, we observed that treatment with the CRT inhibitor significantly decreased cell viability (Fig. 1A), migration (Fig. 1B, C and D) and invasion (Fig. 1E and F). Since we have previously demonstrated that Snail regulates PC cell motility [5], we next analysed Snail expression following CRT treatment, and specifically monitored the level of PKD2 autophosphorylation at Ser<sup>876</sup>. This inhibitor reduced both PKD2 activity and Snail protein levels in a dose-dependent manner (Fig. 2A and B), and a high linear correlation between both parameters was observed (Fig. 2C). By contrast, CRT did not affect Snail expression at a transcriptional level, as assessed by reporter luciferase assays (Fig. 2D). To confirm the specific impact of PDK2 on Snail expression, we measured the protein levels of this transcription factor after silencing this isoform. As shown in Fig. 2E, PKD2 abrogation significantly reduced Snail levels. In order to strength our results, we performed similar experiments in another PKD1-non expressing PC cell line, PC3 [14,15]. As expected, CRT induced significant decreases in cell viability, migration, invasion and Snail protein expression also in these cells (Supplementary Fig. 1A-H). A significant correlation between PKD2 activity and Snail protein levels was also observed (Supplementary Fig. 1I). Moreover, PKD2 silencing reduced Snail protein levels in PC3 cells (Supplementary Fig. 1J), thus ruling out cell-type dependent effects.



**Fig. 1.** PKD inactivation impairs cell viability, migration and invasion in DU145 cells. (A) Cells were treated for 48 h with increasing doses of CRT0066101 (CRT) and cellular viability was measured by MTT assay. (B-D) Wound healing assay and measurement of wound closure area and velocity in cells treated for 48 h with 1  $\mu$ M CRT. (E-F) Invasion capacity using transwell assays in cells treated for 48 h with 2.5  $\mu$ M CRT. For all results, data are shown as the mean  $\pm$  SEM of at least three independent experiments. For migration and invasion assays, pictures are from one representative experiment of three with similar results. Student's *t* test: \* $0.01 < p < 0.05$ ; \*\* $0.001 < p < 0.01$ ; \*\*\* $p < 0.001$ .





**Fig. 2.** PKD inactivation decreases Snail levels, inducing its nuclear exit and proteasomal degradation through GSK3 $\beta$  activation. (A) DU145 cells were treated for 48 h with increasing doses of CRT0066101 (CRT), and levels of Snail, active PKD2 (pPKD2), total PKD2 and tubulin were determined by Western blotting. (B) Quantification of Snail/tubulin fold expression related to untreated control of experiments performed as in A. (C) Correlation between Snail/tubulin and active PKD2/PKD2 of experiments performed as in A. (D) Cells were transfected with the Snail-Luc plasmid, incubated for 48 h in the absence or presence of 2.5  $\mu\text{M}$  CRT, and luciferase activity was measured in cell extracts. (E) Cells were transfected for 48 h with the siControl (siC) or the siPKD2, and levels of Snail, total PKD2, and tubulin were determined by Western blotting. (F) Cells were treated for 48 h with either 1  $\mu\text{M}$  or 2.5  $\mu\text{M}$  CRT0066101 and Snail subcellular location was determined by immunofluorescence. DAPI was used to identify the nuclei. Zoom images from boxed regions are also shown. Scale bar 20  $\mu\text{m}$ . (G) Cells were treated for 24 h with 2.5  $\mu\text{M}$  CRT, incubated in the absence or presence of 10  $\mu\text{M}$  MG132 for the last 12 h, and levels of Snail and tubulin were determined by Western blotting. (H) Quantification of Snail/tubulin fold expression related to untreated control of experiments performed as in G. (I) Cells were treated for 48 h with 2.5  $\mu\text{M}$  CRT, and levels of phospho-GSK-3 $\beta$  (pGSK-3 $\beta$ ), pPKD2, and tubulin were determined by Western blotting. (J) Quantification of pGSK-3 $\beta$ /tubulin fold expression related to untreated control of experiments performed as in I. (K) Correlation between pGSK-3 $\beta$ /tubulin and active PKD2/tubulin of experiments performed as in I. For all results, data are shown as the mean  $\pm$  SEM of at least three independent experiments. For Western blotting and immunofluorescence assays, blots and pictures are from one representative experiment of three with similar results. Student's t test: \*0.01 < p < 0.05; \*\*0.001 < p < 0.01; \*\*\*p < 0.001.

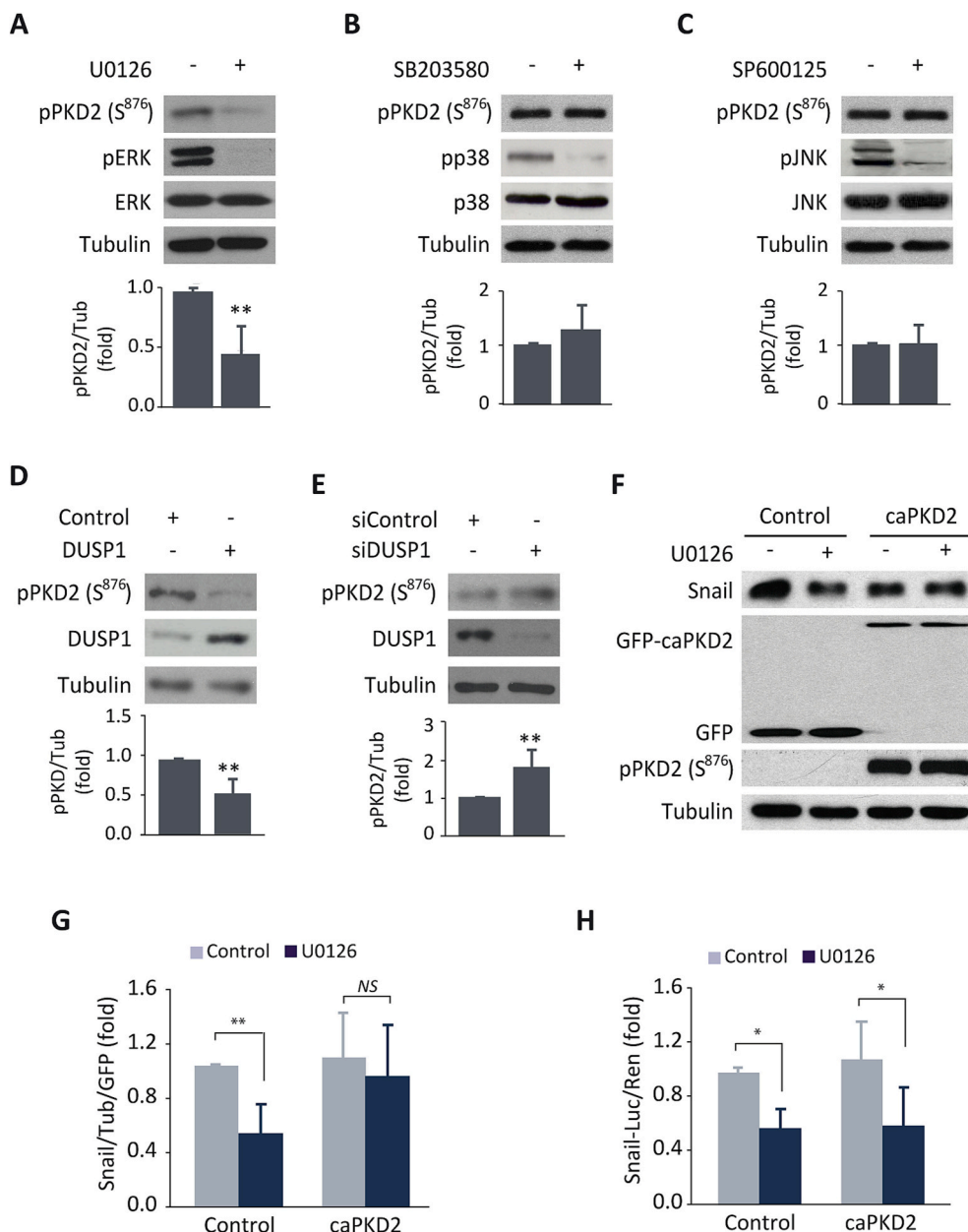
One of the most common molecular mechanisms by which Snail expression is downregulated involves its nuclear export to the cytoplasm and its subsequent proteasome-mediated degradation. To assess whether this was the mechanism involved in Snail regulation by PKD, we analysed the subcellular location of this transcription factor upon treatment with CRT (Fig. 2F). As expected, Snail was mainly nuclear in basal conditions; however, incubation with the PKD inhibitor induced its translocation from the nucleus to the cytoplasm in a dose-dependent manner. Then, we determined the effect of PKD inhibition on Snail proteasome-mediated degradation, observing that the proteasomal inhibitor MG132 hampered the reduction of Snail protein levels induced by CRT (Fig. 2G and H). It is well known that Snail downregulation involves its phosphorylation by GSK-3 $\beta$ , which leads to the nuclear export of this transcription factor and its subsequent proteasome-mediated degradation [31]. In addition, phosphorylation of GSK-3 $\beta$  at residue Ser<sup>9</sup> inactivates this kinase and, therefore, leads to a greater stabilization of Snail [32]. To determine whether this was the mechanism involved in Snail regulation by PKD, we analysed GSK-3 $\beta$  activation upon treatment with CRT. Consistent with the cytoplasmic location

and the decrease in Snail protein levels observed after PKD inhibition, CRT reduced GSK-3 $\beta$  phosphorylation at Ser<sup>9</sup> (Fig. 2I and J) and a high linear correlation between PKD2 and GSK-3 $\beta$  phosphorylation levels was observed (Fig. 2K). Consistently, these CRT effects were also shown in PC3 cells (Supplementary Fig. 1 K and L).

These data altogether demonstrate that PKD activity stabilizes Snail by inducing its nuclear location through a mechanism involving the inactivation of GSK-3 $\beta$ , and consequently, upregulates migration and invasion in PC cells.

### 3.2. PKD2 activity mediates the effects of ERK signalling pathway on Snail expression in PC cells

We have recently shown that inhibition of both ERK and JNK pathways decreases motility of PC cells through Snail reduction [5]. Therefore, we next studied whether these MAPKs could be involved in the mechanism employed by PKD to regulate Snail protein levels. Importantly, we observed that inhibition of the ERK cascade with the MEK inhibitor U0126 significantly reduced PKD2 activity in both DU145 cells



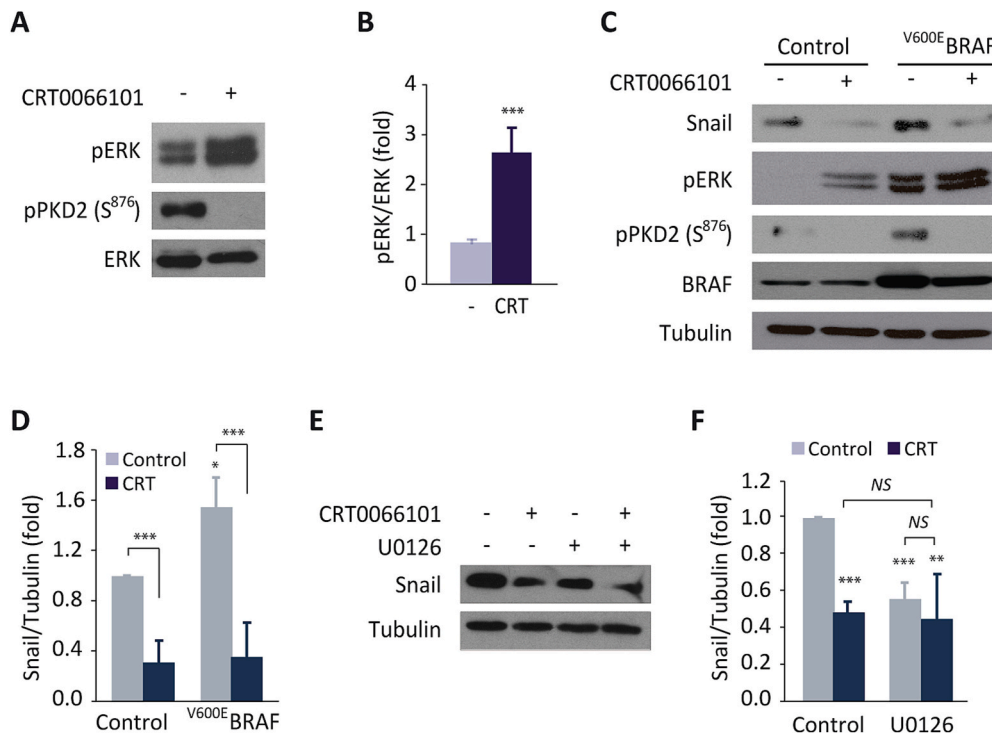
**Fig. 3.** PKD2 activity mediates the effects of ERK signaling pathway on Snail expression in DU145 cells. (A) Cells were treated for 48 h with 20  $\mu$ M U0126, and levels of active PKD2 (pPKD2), active ERK (pERK), total ERK, and tubulin were determined by Western blotting. (B) Cells were treated for 48 h with 1  $\mu$ M SB203580, and levels of pPKD2, active p38 (pp38), total p38, and tubulin were determined by Western blotting. (C) Cells were treated for 48 h with 10  $\mu$ M SP600125, and levels of pPKD2, active JNK (pJNK), total JNK, and tubulin were determined by Western blotting. (D) Cells were transfected for 48 h with a control vector (Control) or a vector encoding DUSP1 (DUSP1), and levels of pPKD2, DUSP1, and tubulin were determined by Western blotting. (E) Cells were transfected for 48 h with the siControl or the siDUSP1, and levels of pPKD2, DUSP1, and tubulin were determined by Western blotting. (F) Cells were transfected with a GFP-constitutively active PKD2 mutant (caPKD2) and incubated for 48 h with 20  $\mu$ M U0126. Levels of Snail, GFP, pPKD2 and tubulin were determined by Western blotting. (G) Quantification of Snail/tubulin/GFP fold expression related to untreated control of experiments performed as in F. (H) Cells were transfected with the Snail-Luc and the caPKD2 plasmids, incubated for 48 h with 20  $\mu$ M U0126, and luciferase activity was measured. For all results, bar graphs show the mean  $\pm$  SEM of at least three independent experiments. For Western blotting assays, blots are from one representative experiment of three with similar results. Student's t test: \*0.01 < p < 0.05; \*\*0.001 < p < 0.01; \*\*\*p < 0.001.

(Fig. 3A) and PC3 cells (Supplementary Fig. 2A). However, no significant variations in PKD2 autophosphorylation were observed in these two PC cell lines in the presence of SB203580 or SP600125, inhibitors of p38MAPK or JNK, respectively (Fig. 3B and C, and Supplementary Fig. 2B). The efficiency of inhibition of each MAPK activity by either U0126, SB203580 or SP600125 was confirmed by measuring the levels of ERK, p38MAPK and JNK phosphorylation in cells incubated with these compounds, respectively (Fig. 3A, B and C). In addition, the connection between ERK and PKD2 activities was also confirmed by either overexpression (Fig. 3D) or silencing (Fig. 3E) of the ERK-inactivating phosphatase, DUSP1, showing that PKD2 activity was also modulated by this phosphatase. DUSP1 overexpression and silencing efficiency was tested by measuring its protein levels (Fig. 3D and E). To test whether PKD2 activity mediated the effects of MEK/ERK pathway on Snail expression [5], we overexpressed a constitutively active PKD2 mutant (caPKD2) and analysed Snail protein levels in the presence or absence of U0126. Active PKD2 prevented the downregulation of Snail induced by MEK/ERK cascade inhibition (Fig. 3F and G). By contrast, caPKD2 did not affect the reduced transcription of Snail achieved by the MEK inhibitor (Fig. 3H). These results demonstrate that PKD2 activity mediates, at least in part, the effects of ERK pathway on Snail protein expression. In order to define whether Snail expression was also regulated by PKD through ERK activation, we next analysed active ERK levels in CRT-treated cells, observing that PKD inhibition significantly increased ERK activity (Fig. 4A and B). These data suggested that PKD does not regulate Snail levels by affecting ERK pathway. To confirm this hypothesis, we overexpressed a constitutively active BRAF mutant ( $V^{600E}$ BRAF), to hyperactivate MEK/ERK pathway, and analysed Snail protein levels in the presence or absence of CRT. As expected, BRAF mutant activated PKD2 and increased levels of Snail downstream target (Fig. 4C and D). Moreover, inhibition of PKD decreased Snail levels independently of the presence of mutated BRAF (Fig. 4C and D). In addition, the combination of CRT with the MEK inhibitor U0126 did not lead to further reduction of Snail levels compared to that obtained upon single treatments (Fig. 4E and F).

All these data, together with those shown in Fig. 2, demonstrate that the signalling pathway that regulates Snail protein expression involves the ERK-mediated activation of PKD2, which in turn inactivates GSK-3 $\beta$  and favours Snail nuclear location and stabilization.

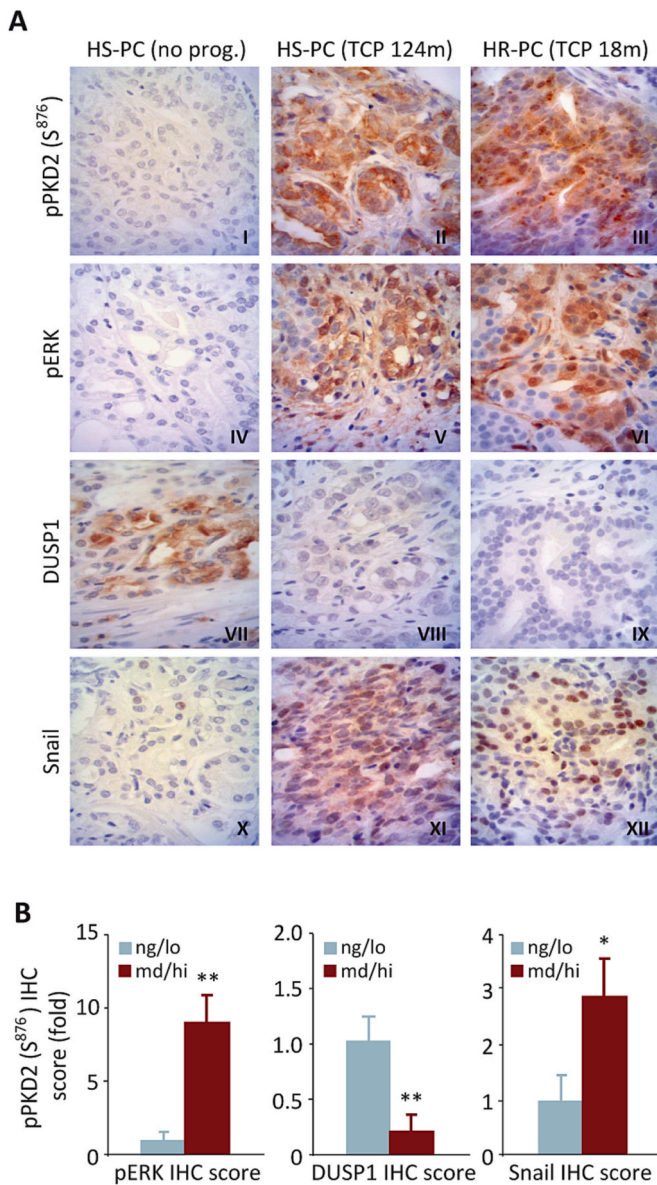
### 3.3. PKD2 activity correlates with active ERK and Snail levels, and is associated with clinical poor prognostic factors in PC patients

To investigate the clinical relevance of our results, we next analysed the levels of active PKD2, active ERK, DUSP1 and Snail expression in samples from patients diagnosed with either BPH or advanced PC, and we studied their correlation with tumoral progression. It is important to point out that there are wide evidences of absence or nearly undetectable expression of PKD1 in advanced prostate tumours [10–13]. Therefore, in the analysis of patient samples, the signal obtained with the phospho-antibody used to detect both active PKD1 and PKD2, mostly corresponds to PKD2 autophosphorylation. Having this in mind, we first measured the levels of active PKD2 in BPH samples by immunostaining, observing no signal for phosphorylated PKD2 (data not shown). This result correlates well with our previous findings showing low expression of both Snail and active ERK, together with high DUSP1 expression levels in BPH biopsies [3,5]. Then, we compared immunostaining of phosphorylated active PKD2 and ERK, together with DUSP1 and Snail levels in samples from patients with different stages of advanced PC: hormone responsive-PC (HS-PC) with no clinical progression and no exitus; HS-PC with long TCP and exitus; and HR-PC with short TCP and exitus. Active PKD2 and active ERK signals were negative to low in samples from HS-PC patients without clinical progression and no exitus (Fig. 5A–I,IV), whereas high signal of these markers was detected in samples from patients with HS-PC (TCP 124 m) or HR-PC (TCP 18 m) and exitus as outcome (Fig. 5A–I,II,III,V,VI). Conversely, samples from HS-PC patients with no clinical progression showed a moderate to high DUSP1 signal (Fig. 5A–VII), while expression of this phosphatase was negative to low in samples from HS-PC and HR-PC with clinical progression (Fig. 5A–VIII,IX). Additionally, the levels of Snail were lower in



**Fig. 4.** The constitutive activation of MEK/ERK cascade does not mediate the effects of PKD2 activity on Snail expression in DU145 cells. (A) Cells were treated for 48 h with 2.5  $\mu$ M CRT0066101 (CRT), and levels of active ERK (pERK), active PKD2 (pPKD2), and total ERK were determined by Western blotting. (B) Quantification of pERK/ERK fold expression related to untreated control of experiments performed as in A. (C) Cells were transfected with a constitutively active BRAF mutant ( $V^{600E}$ BRAF) and incubated for 48 h with 2.5  $\mu$ M CRT. Levels of Snail, pERK, pPKD2, total BRAF, and tubulin were determined by Western blotting. (D) Quantification of Snail/tubulin fold expression related to untreated control of experiments performed as in C. (E) Cells were treated for 48 h with 20  $\mu$ M U0126, incubated in the absence or presence of 2.5  $\mu$ M CRT for the last 24 h, and levels of Snail and tubulin were determined by Western blotting. (F) Quantification of Snail/tubulin fold expression related to untreated control of experiments performed as in E. For all results, bar graphs show the mean  $\pm$  SEM of at least three independent experiments. For Western blotting assays, blots are from one representative experiment of three with similar results. Student's t test: \*0.01 < p < 0.05; \*\*0.001 < p < 0.01; \*\*\*p < 0.001.





**Fig. 5.** PKD2 activity is increased in human advanced PC and correlates with active ERK and Snail expression levels. (A) Immunohistochemical analysis of expression levels of active pPKD2 (pPKD2; I-III), active ERK (pERK; IV-VI), DUSP1 (VII-IX) and Snail (X-XII) in human samples from hormone responsive-PC (HS-PC) with no clinical progression (no prog.), HS-PC with long time to clinical progression (TCP) or hormone refractory-PC (HR-PC) with short TCP. Micrographs were taken at 200 $\times$  magnification and show serial sections from the same gland stained with one of the four used antibodies. (B) Correlations of immunohistochemical scores for pPKD2 with pERK, DUSP1 and Snail levels in samples ranged into two categories based on the staining pattern of the majority of tumor cells in the whole section (negative/low (ng/lo); moderate/high (md/hi)). The statistical analysis was performed with One-way ANOVA and Dunnett's multiple comparison test. Asterisks show the statistical significance of differences between groups for each marker (\*0.01 < p < 0.05; \*\*0.001 < p < 0.01).

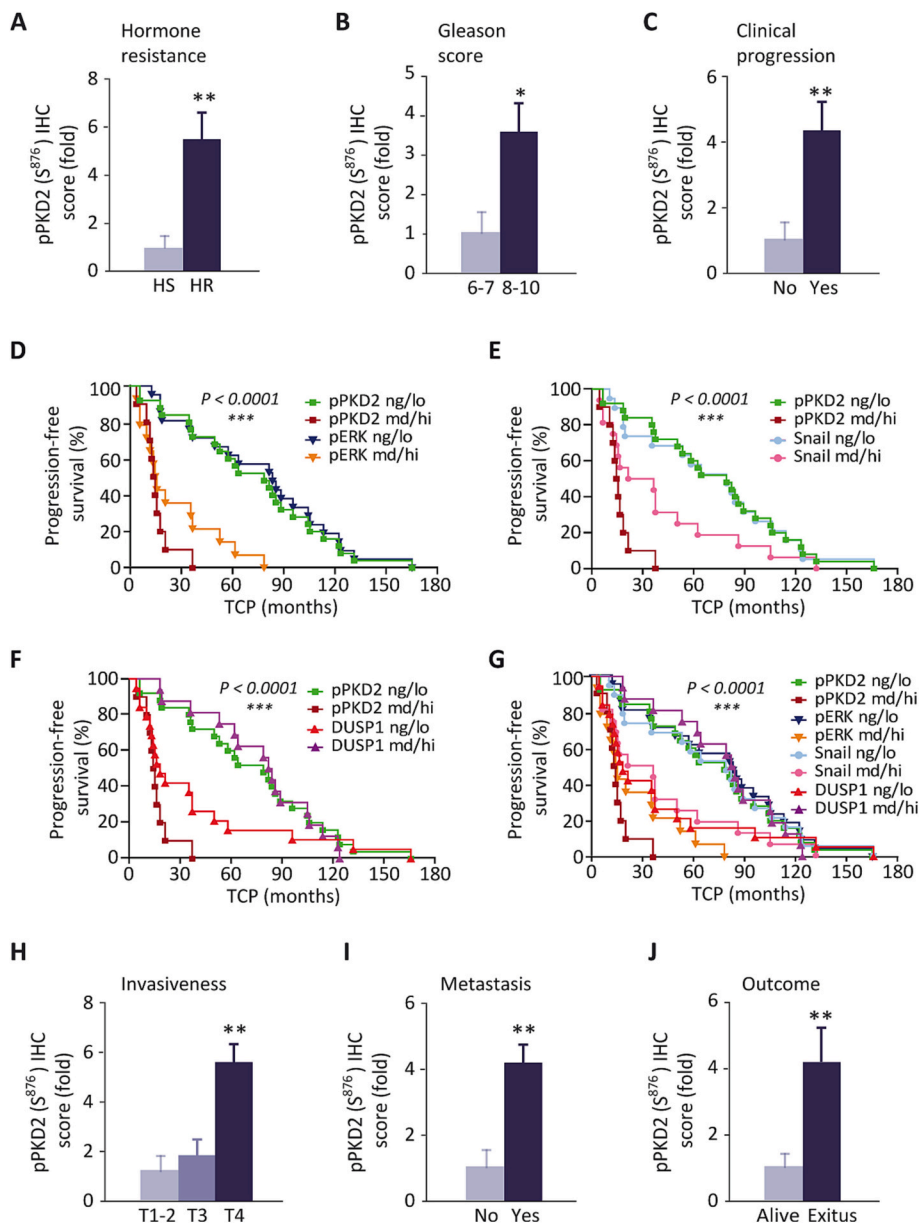
HS-PC patients without clinical progression (Fig. 5A-X) than in those with HS-PC (TCP 124 m; Fig. 5A-XI) or HR-PC (TCP 18 m; Fig. 5A-XII). The analysis of these observations was confirmed by the Pearson's Test, showing that active PKD2 levels directly correlated with both active ERK and Snail expression levels, and inversely correlated with DUSP1 levels (Fig. 5B).

We finally studied the interrelation of PKD2 activity with the most

important PC clinical prognostic factors. Our results evidenced significant direct correlations of high levels of active PKD2 with hormone resistance (Fig. 6A), Gleason score (Fig. 6B) and clinical progression (Fig. 6C). We also observed highly significant correlations regarding the disease progression when comparing the levels of active PKD2 with active ERK (Fig. 6D), Snail expression (Fig. 6E) and DUSP1 expression (Fig. 6F). Thus, shorter TCP were related with higher levels of active PKD2, active ERK (Fig. 6D), and Snail (Fig. 6E), as well as with lower DUSP1 expression (Fig. 6F). Moreover, the combined pattern pPKD2<sup>high</sup>/pERK<sup>high</sup>/Snail<sup>high</sup>/DUSP1<sup>low</sup> was strongly related with overall TCP (Fig. 6G). Consistently, we observed a direct robust correlation of active PKD2 with parameters related to a more advanced disease, such as the existence of invasiveness (Fig. 6H), metastasis (Fig. 6I) and with the exitus rate (Fig. 6J). Collectively, the results in human PC samples reveal the existence of a direct connection between the activity of PKD2 and ERK with the levels of Snail. In addition, these results support our data in PC cell lines, reinforcing that ERK signalling pathway regulates Snail expression through PKD2 activity in PC. Moreover, these data pinpoint that the pPKD2-pERK-Snail-DUSP1 ratio is an important biomarker profile for PC prognosis.

#### 4. Discussion

The role played by PKD isoforms in PC is far to be clear, since it has been reported that they can promote or suppress tumour growth and/or progression [8,9,11,14,16,17]. The main processes involved in tumour progression are uncontrolled cell migration and invasion, which are related to induced epithelial-to-mesenchymal transition (EMT) [33]. Multiple evidences indicate that PKD expression could regulate EMT in PC epithelial cells through diverse molecular mechanisms. Thus, whereas PKD1 is associated with decreased cellular motility [10,16] and inhibition of EMT by decreasing Snail nuclear location [11], PKD2 appears to act in the opposite manner, positively regulating PC cell migration by increasing matrix metalloproteinases expression [17]. In addition, various studies provide indirect evidence of the anti-migratory role of PKD inhibition in PC cells [19–22], although they are not conclusive because the use of non-selective inhibitors, that target a few additional kinases related to MAPK pathways [20,21]. Bearing this in mind, using the selective pan-PKD inhibitor, CRT0066101, here we demonstrate that PKD activation regulates cell viability and invasiveness in PKD1-non expressing PC cells. Our results show that PKD inactivation by CRT inhibitor decreases cell migration and invasion, in agreement with a recent report of Varga *et al* [15]. Moreover, in this study we demonstrate for the first time that PKD2 activity is directly correlated with the expression levels of the EMT-inducer transcription factor, Snail. These data are consistent with those observed in lung cancer, showing that high expression of PKD2 is associated with EMT promotion and enhanced cell motility, as well as with poor prognosis and cancer progression in lung adenocarcinoma patients [34]. Moreover, the anti-proliferative and anti-invasive role of PKD inhibition by CRT has been demonstrated in other tumour contexts where PKD2 is the dominant isoform expressed, such as bladder and colon cancer cells [35,36]. On the other hand, our study demonstrates that the mechanism by which PKD2 inhibition decreases Snail expression involves the activation of GSK-3 $\beta$ , as well as the nuclear exportation and the proteasome-mediated degradation of this transcription factor, similarly to our previous data after MEK/ERK blockage [5]. These data are consistent with previous reports showing that PKD inhibits GSK-3 $\beta$  activity in breast cancer [37], hepatocellular carcinoma [38], and endothelial cells [39], and suggest that GSK-3 $\beta$  activation by CRT could be a novel strategy to fight cancer. Additionally, we show that PKD2 autophosphorylation depends on ERK activation and overexpression of a PKD2 constitutively active mutant rescues the dampened Snail expression caused by the specific inactivation of ERK pathway. All these data demonstrate the existence of a direct connection between ERK and PKD2 activities, in which inhibition of ERK signalling cascade inactivates PKD2 and



**Fig. 6.** PKD2 activity is associated with clinical poor prognostic factors in PC patients. (A) Immunohistochemical (IHC) score for active PKD2 (pPKD2) in samples from patients either with hormone responsive (HS) or hormone refractory (HR) PC. (B) IHC score for pPKD2 in samples from patients ranged into two categories based on their Gleason score. (C) IHC score for pPKD2 in samples from patients ranged into two categories based on their clinical progression. (D-G) Progression-free survival of patients showing IHC score for pPKD2/active ERK (pERK), pPKD2/Snail, pPKD2/DUSP1 or pPKD2/pERK/Snail/DUSP1 in samples ranged into two categories based on the staining pattern of the majority of tumor cells in the whole section (negative/low (ng/lo); moderate/high (md/hi)). (H) IHC score for pPKD2 in samples from patients ranged into three categories based on their invasiveness. (I) IHC score for pPKD2 in samples from patients ranged into two categories based on the existence of metastasis. (J) IHC score for pPKD2 in samples from patients either alive or dead. The statistical analysis was performed with One-way ANOVA and Dunnett's multiple comparison test. Asterisks show the statistical significance of differences between the groups (\*0.01 < p < 0.05; \*\*0.001 < p < 0.01; \*\*\*p < 0.001). TCP, Time to clinical progression.

decreases Snail protein levels. Several studies have shown that PKD inhibition impairs proliferation and tumour growth through an ERK-dependent mechanism in cancer models of colon [36], breast [40], or in head and neck squamous cell carcinoma [41]. Focusing on PC, it has been previously shown that PKD2 interacts with ERK in cellular models, and silencing of this isoform decreases the ERK activation induced by PKD agonist PMA [42]. By contrast, here we evidence that PKD inhibition significantly activates ERK, and the reduced Snail levels induced by CRT are not affected by the constitutive activation of MEK/ERK pathway. All these data reveal a complex connection between PKD2 and ERK kinases and reinforce our demonstration that ERK/PKD2 pathway regulates Snail in PC cells. On the other hand, it is noteworthy that JNK activity does not modulate PKD2 autophosphorylation, despite the fact that we have previously shown that this signalling cascade regulates cell migration and invasion through the modulation of Snail content and location in PC cells [5]. Moreover, p38MAPK does not affect PKD2 activity either, consistently with our previous data showing that this MAPK does not regulate Snail expression [5] and those from others demonstrating no interaction between the pathways of both kinases in our cells [42].

Interestingly, in this study we also show for the first time, higher levels of active PKD2 in samples from patients with advanced PC, compared to those developing less aggressive tumours. Moreover, we demonstrate that PKD2 activity is related to poor prognostic factors, being higher in PC patients with clinical progression and exitus as final outcome, than in those who survived because their tumours did not clinically progress. Although we are aware that the antibody used in this study recognizes the autophosphorylated forms of PKD1 and PKD2, it is widely accepted that PKD1 is weakly expressed or even absent in advanced PC [10–13], as well as in late stages of other tumours, including gliomas and breast, gastric, bladder and colon cancers [35,36,43–45]. Therefore, all these data strongly support that PKD2 is the isoform detected in this study. Recently, no higher autophosphorylation levels at Ser<sup>910</sup>/Ser<sup>876</sup> have been observed in human PC samples compared to normal tissues [42]. This apparent contradiction with our results can be explained by the different characteristics of the analysed samples. Thus, the specimens from the study of Xu *et al* have Gleason scores between 1 and 5 [42], while those from our samples are higher than 6. In fact, we also observe low phospho-PKD2 levels in samples with Gleason score between 6 and 7, while these levels significantly



increase as this index rises. Altogether these results demonstrate that PKD2 autophosphorylation is directly correlated with Gleason score. In addition, we also show that PKD2 activity is associated with most of the PC malignancy factors, including the androgenic independence, the clinical progression, the degree of invasiveness and metastasis, as well as the outcome. These data are extraordinarily important since no previous studies on this matter are available for prostate tumours. In accordance with our results, the percentage of active PKD2-positively stained cells is significantly higher in human hepatocellular carcinoma compared to normal tissues, and this increase is correlated with the recurrence status of these patients [38]. Additionally, our results are consistent with those observed in lung cancer and glioblastoma, demonstrating a high correlation between the number of PKD2-positive tumour cells with tumour grading and poor prognosis [34,45]. In conclusion, all these data in clinical samples strongly suggest that PKD2 autophosphorylation is a very reliable PC malignancy biomarker, that might be an essential common determinant for tumour progression in a variety of PKD1-lacking tumours. On the other hand, although our data show a high correlation between PKD2 activity, Snail expression and the induction of migration and invasion, given that the cell lines used in our study also express PKD3, we cannot discard additional contribution of this isoform in these processes. In this sense, it has been previously demonstrated that PKD3 promotes PC cell survival and migration [14,15], by regulating the expression of various metastasis-associated genes, among which Snail is not included [17]. In addition, PKD3 expression is more frequent and high in advanced human PC, although this increase does not correlate with the Gleason index or with the degree of malignancy [14]. However, our results demonstrating that active PKD2 levels are associated to these and other important risk prognostic parameters, highlight the idea that PKD isoforms might play stage-specific roles in PC progression. For these reasons, further studies are needed to address the relative contribution of PKD3 activity to PC progression.

In summary, our results in advanced human prostate tumours confirm and give relevance to the data obtained in cell models, since they show the existence of a positive correlation between PKD2 activation, active ERK and Snail levels. Consequently, we observed that the expression pattern pPKD2<sup>high</sup>/pERK<sup>high</sup>/Snail<sup>high</sup> in patient samples is indicative of poor prognostic disease factors, such as clinical progression, invasiveness and metastasis. All these results suggest that the combined monitoring of PKD2 phosphorylation at Ser<sup>876</sup> and ERK activity may be a viable and very reliable diagnostic strategy for advanced PC. Moreover, our results provide a rationale for designing new treatments based on the reduction of PKD2 and ERK activation for advanced prostate tumours.

### Statement of ethics

The study involving human specimens was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Hospital Universitario de Getafe (A17–11 of 10/27/2011).

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### CRediT authorship contribution statement

All authors have read and agreed to the published version of the manuscript. Their individual contribution to the paper is detailed below. Darío Cilleros-Rodríguez: Methodology, Investigation, Formal analysis. María Val Toledo-Lobo: Methodology, Investigation, Formal analysis,

Resources. Desirée Martínez-Martínez: Methodology, Investigation. Pablo Baquero: Funding acquisition, Resources, Writing—review and editing. Javier C. Angulo: Resources, Writing—review and editing. Antonio Chiloeches: Conceptualization, Funding acquisition, Methodology, Resources, Writing—review and editing, Supervision. Teresa Iglesias: Conceptualization, Funding acquisition, Resources, Writing—review and editing. Marina Lasa: Conceptualization, Funding acquisition, Methodology, Resources, Writing—original draft, review and editing, Supervision.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Data availability

Data will be made available on request.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbdis.2023.166851>.

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