PKD2 and PKD3 promote prostate cancer cell invasion by modulating NF-κB- and HDAC1-mediated expression and activation of uPA

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Summary

Although protein kinase D3 (PKD3) has been shown to contribute to prostate cancer cell growth and survival, the role of PKD in prostate cancer cell motility remains unclear. Here, we show that PKD2 and PKD3 promote nuclear factor kappa B (NF-κB) signaling and urokinase-type plasminogen activator (uPA) expression/activation, which are crucial for prostate cancer cell invasion. Silencing of endogenous PKD2 and/or PKD3 markedly decreased prostate cancer cell migration and invasion, reduced uPA and uPA receptor (uPAR) expression and increased plasminogen activator inhibitor-2 (PAI-2) expression. These results were further substantiated by the finding that PKD2 and PKD3 promoted the activity of uPA and matrix metalloproteinase 9 (MMP9). Furthermore, depletion of PKD2 and/or PKD3 decreased the level of binding of the p65 subunit of NF-κB to the promoter of the gene encoding uPA (*PLAU*), suppressing transcriptional activation of uPA. Endogenous PKD2 and PKD3 interacted with inhibitor of NF-κB (IκB) kinase β (IKKβ); PKD2 mainly regulated the phosphorylated IKK (pIKK)-phosphorylated IκB (pIκB)-IκB degradation cascade, p65 nuclear translocation, and phosphorylation of Ser276 on p65, whereas PKD3 was responsible for the phosphorylation of Ser536 on p65. Conversely, inhibition of uPA transactivation by PKD3 silencing was rescued by constitutive Ser536 p65 phosphorylation, and reduced tumor cell invasion resulting from PKD2 or PKD3 silencing was rescued by ectopic expression of p65. Interestingly, PKD3 interacted with histone deacetylase 1 (HDAC1), suppressing HDAC1 expression and decreasing its binding to the uPA promoter. Moreover, depletion of HDAC1 resulted in recovery of uPA transactivation in PKD3-knockdown cells. Taken together, these data suggest that PKD2 and PKD3 coordinate to promote prostate cancer cell invasion through p65 NF-κB- and HDAC1-mediated expression and activation of uPA.

Key words: PKD, Prostate cancer, uPA, NF-κB, HDAC1, Invasion

Introduction

Although tremendous efforts have been made to improve the effectiveness of treatment, prostate cancer remains the second leading cause of cancer-related deaths among men in the USA. Morbidity and mortality primarily result from the growth of metastatic tumors in distant organs. Multiple steps are involved in the metastatic process. In the initial step, the extracellular matrix (ECM) surrounding the tumor is remodeled, allowing tumor cells to invade across the basement membrane and into circulation. One of the most important molecules involved in the ECM remodeling process is urokinase plasminogen activator (uPA), a serine protease that initiates the activation of ECM-degrading enzymes and, through association with its receptor (uPAR), modulates various biological functions, including cell migration, angiogenesis, differentiation and wound healing (Dass et al., 2008). However, the mechanisms that regulate uPA expression during metastasis have not been fully defined.

Many studies have suggested that nuclear factor kappa B (NF-κB) has a pivotal role in promoting the invasive and metastatic potential of prostate cancer. In contrast to LNCaP cells, a less invasive prostate cancer cell line, PC-3 and DU145 cells exhibit constitutive activation of the NF-κB DNA-binding activity (Palayoor et al., 1999). In addition, overexpression of NF-κB in LNCaP and DU145 cells significantly increases the expression of prostate-specific antigen (PSA), which is closely associated with the progression of prostate cancer (Chen and Sawyers, 2002). Conversely, inhibition of NF-κB prevents tumor angiogenesis, invasion and metastasis in PC-3M xenografts in nude mice (Huang et al., 2001). Nevertheless, the signaling pathways upstream of NF-κB that lead to prostate cancer invasion and metastasis have not yet been illustrated.

Protein kinase D (PKD) belongs to a family of serine/threonine protein kinases that is now classified as a subfamily of the Ca^{2+}/calmodulin-dependent kinase (CaMK) superfamily. Three members of the PKD family have been identified to date, namely, PKD1 (PKCμ), PKD2 and PKD3 (PKCν) (Wang, 2006). PKD has been implicated in various biological processes, such as cell proliferation (McEneaney et al., 2010; Papazyan et al., 2008; Wang, 2006), survival (Arun et al., 2011; Song et al., 2009), cell migration (Eiseler et al., 2010; Peterburs et al., 2009),

differentiation (Jadali and Ghazizadeh, 2010; Kleger et al., 2011), inflammation (Kim et al., 2010b; Park et al., 2009) and vesicle transportation (Chen et al., 2009; Hausser et al., 2005; Lu et al., 2007). The role of PKDs in cancer invasion/metastasis has been increasingly recognized, and, of the three PKD isoforms, PKD1 has been the focus of most studies in this context. In breast cancer cells, the expression of PKD1 has been shown to be inversely correlated with invasive potential, impeding this process through downregulation of the expression of the matrix metalloproteinases (MMPs) MMP2, MMP7, MMP9, MMP10, MMP11, MMP13 and MMP14 (also known as membrane-type 1 MMP, or MT1-MMP) (Eiseler et al., 2009). Furthermore, overexpression of PKD1 increases aggregation, but reduces motility and invasiveness of prostate cancer cell lines through interaction with and phosphorylation of E-cadherin (Jaggi et al., 2005; Syed et al., 2008). In our previous study, we found that PKD3 levels were substantially increased in human prostate tumors compared with those in normal tissues, as revealed by immunohistochemistry. Moreover, PKD3 exhibited a marked increase in nuclear localization in tumor tissues, correlating with tumor grade. Increased protein expression and nuclear accumulation of PKD3 were observed in the more invasive and metastatic PC-3 and DU145 cell lines compared to the less aggressive LNCaP cell line, and this was shown to contribute to the growth and survival of prostate cancer cells (Chen et al., 2008). However, the exact role of PKD2 and PKD3 in tumor cell invasion has not been characterized. Thus, the primary goal of this study was to investigate the function and regulatory mechanisms of PKD2- and PKD3-mediated pathways in prostate cancer cell invasion.

Results

Knockdown of PKD2 and PKD3 inhibits the migration and invasion of prostate cancer cells

Our previous study demonstrated that PKD3 promotes prostate cancer cell growth and survival (Chen et al., 2008). In addition, we found that PKD3 expression was increased in more invasive prostate cancer cell lines (i.e. expression of PKD3 in PC-3 and DU145 cells is higher than in LNCaP cells), suggesting that there is a positive role for PKD3 in regulating the aggressiveness of prostate cancer cells. However, the involvement of PKD3 in prostate cancer cell invasion has not been determined. Here, we analyzed whether PKD2 and PKD3, the two predominant PKD isoforms expressed in PC-3M, PC-3 and DU145 cells, have a role in the migration and invasion of prostate cancer cells. In DU145 and PC-3M cells, small interfering RNA (siRNA)-mediated depletion of PKD2 or PKD3 significantly decreased cell migration, with depletion of PKD3 resulting in a more substantial decrease than depletion of PKD2 (Fig. 1A,B). Analysis of cell invasion using Matrigel-coated filters in transwell chambers demonstrated that knockdown of PKD2 or PKD3 inhibited the invasion of PC-3M cells. Moreover, depletion of both PKD2 and PKD3 resulted in greater inhibitory effects

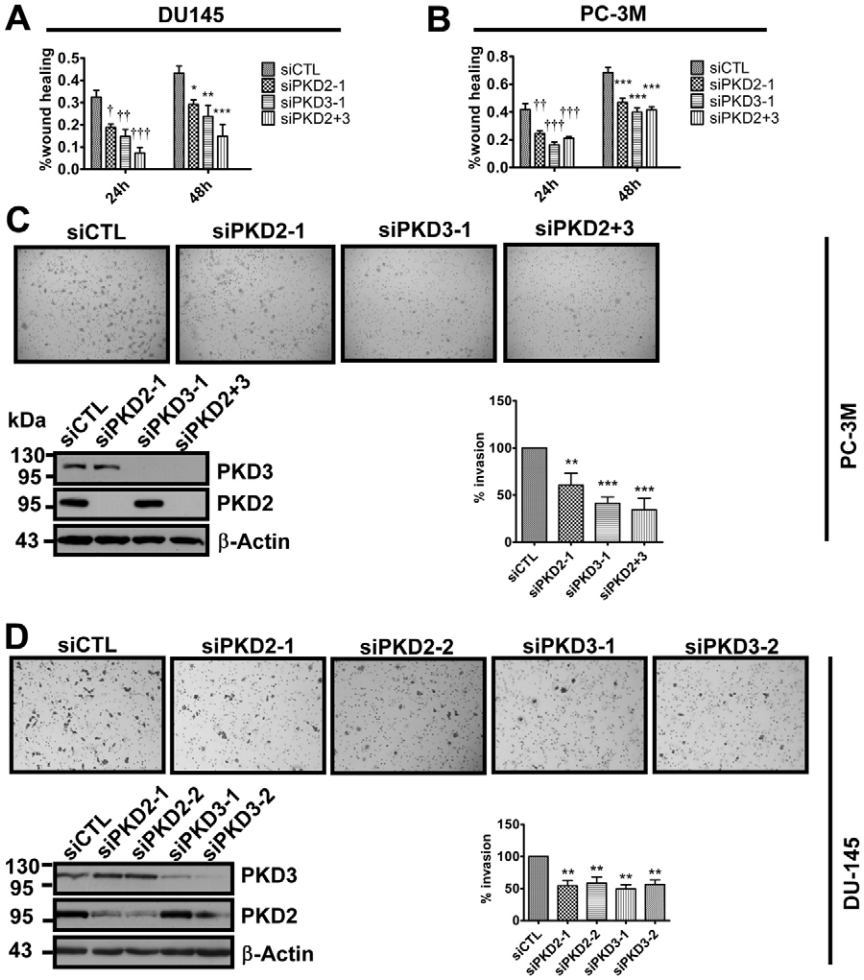

Fig. 1. Knockdown of PKD2 or PKD3 decreases prostate cancer cell migration and invasion. DU145 (**A**) and PC-3M (**B**) cells were transiently transfected with control (siCTL), PKD2 (siPKD2-1), PKD3 (siPKD3-1), or both PKD2 and PKD3 (siPKD2+3) siRNAs. After 24 hours, the cell monolayer was scratched. Cells were incubated for an additional 48 hours. Wound healing was analyzed by comparing the wound healing rate [% wound healing=(Initial wound width − current wound width)/Initial wound width] at two different intervals. Data represent the mean±s.e.m. of three independent experiments and were analyzed by two-way ANOVA with multiple comparisons (PKD-knockdown effect and time interval effect), followed by Bonferroni post hoc test for significance. *$P<0.05$; **$P<0.01$, and ***$P<0.001$ versus siCTL (48 hours) cells; $^{\dagger}P<0.05$; $^{\dagger\dagger}P<0.01$ and $^{\dagger\dagger\dagger}P<0.001$ versus siCTL (24 hours) cells. PC-3M (**C**) and DU145 (**D**) cells were transiently transfected with siCTL, PKD2 siRNAs (siPKD2-1, siPKD2-2), PKD3 siRNAs (siPKD3-1, siPKD3-2), or siPKD2+3 as indicated. At 24 hours after transfection, cells were seeded into transwell chambers and cultured for an additional 24 hours. Upper panels: invasion potential through Matrigel-coated transwells was monitored by Crystal Violet staining (see Materials and Methods). Lower left panels: western blotting results indicating sufficient silencing of PKD2 and PKD3 protein by transient siRNA transfection. Lower right panels: the percentage invasion is plotted as the number of cells invading through Matrigel-coated transwell chambers divided by the number of cells invading through uncoated chambers. The percentage invasion of other groups was normalized to siCTL-transfected cells, set at 100%. Data represent the mean±s.e.m. of three independent experiments and were analyzed by one-way ANOVA with multiple comparisons, followed by Dunnett post hoc test for significance versus siCTL. **$P<0.01$ and ***$P<0.001$ versus siCTL.

(Fig. 1C). To demonstrate the absence of possible off-target effects, we used two siRNAs for each isoform, targeting different regions of the *PKD2* or *PKD3* mRNA, and monitored the effects of these siRNAs on the invasion of DU145 cells. Consistent with our above results, the invasion potential of DU145 cells was significantly reduced by two different siRNA targeting PKD2 or PKD3 (Fig. 1D). These results suggest that both PKD2 and PKD3 contribute to prostate cancer cell migration and invasion.

PKD2 and PKD3 mediate the expression of invasion- and metastasis-related genes in the uPA-uPAR and MMP pathways

Given that PKD2 and PKD3 were shown to be crucial for prostate cancer cell migration/invasion, we sought to identify the genes responsible for PKD2- and PKD3-induced invasion. Our results demonstrated that depletion of PKD2 (siPKD2-1), PKD3 (siPKD3-1), or both PKD2 and PKD3 (siPKD2+3) in PC-3M cells led to significant downregulation of activin A, MT1-MMP, uPA and uPAR, and upregulation of plasminogen activator inhibitor-2 (PAI-2) at the mRNA level compared with a nontargeting siRNA control (siCTL); no significant difference in the expression of osteopontin or vascular endothelial growth factor (VEGF) was observed (Fig. 2A). Among these invasion/metastasis-related genes, we focused on those involved in the uPA-uPAR and MMP systems and further investigated changes in the expression of these targets at the protein level by western blotting. Protein levels of uPA, uPAR and MT1-MMP were significantly downregulated in PC-3M (Fig. 2C) and DU145 cells (supplementary material Fig. S2A) after silencing of PKD2 or PKD3 using two different siRNAs, also verifying the absence of off-target effects for these PKD2 or PKD3 siRNAs. Because phorbol 12-myristate 13-acetate (PMA) has been shown to activate PKC and PKD family members (Wang, 2006) and upregulate uPA-, uPAR- and MT1-MMP-encoding mRNA expression in PC-3M cells (supplementary material Fig. S1), we used PMA to induce the expression PKD and the uPA system proteins. Transfection with siPKD2, siPKD3 or siPKD2+3 resulted in decreased expression of uPA, uPAR and MT1-MMP and increased expression of PAI-2 compared with transfection with siCTL in PC-3M (Fig. 2B) and DU145 (supplementary material Fig. S2C) cells, both with and without PMA stimulation. Moreover, given that epidermal growth factor (EGF) has been shown to upregulate uPA-uPAR signaling (Festuccia et al., 2005; Amos et al., 2010) and initiate prostate cancer cell invasion (Jarrard et al., 1994), and tumor necrosis factor (TNF)-α (pivotal in inflammation and invasion interactions) (Stock et al., 2008) has been shown to upregulate expression of the gene encoding uPA (*PLAU,* hereafter referred to as the uPA gene) (Kim et al., 2010a; Guerrini et al., 1996), we used EGF and TNF-α treatments to determine whether PKD2 or PKD3 regulate uPA, uPAR and MT1-MMP expression through these two physiological agonists. As shown in Fig. 2D and supplementary Fig. S2B, TNF-α significantly induced the expression of uPA and MT1-MMP, and this effect was dramatically reduced by silencing of PKD2 or PKD3 in both cell lines. Similarly, EGF-induced MT1-MMP expression was also

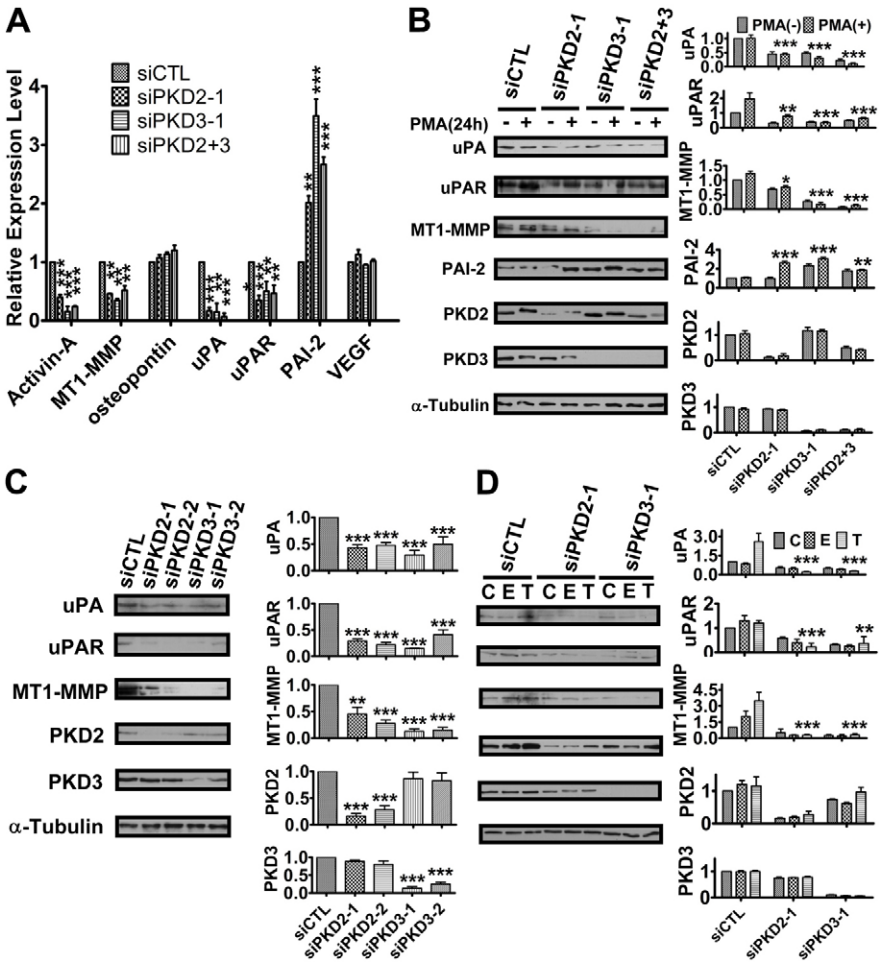

Fig. 2. PKD2 and PKD3 are crucial for expression of invasion- and metastasis-related genes in the uPA-uPAR and MMP pathways. (**A**) PC-3M cells were transiently transfected with control siRNA (siCTL), PKD2 siRNA (siPKD2-1), PKD3 siRNA (siPKD3-1), or both (siPKD2+3). After 48 hours, mRNA levels of indicated pro-invasion or metastasis genes were analyzed by RT-qPCR. Data represent the mean±s.e.m. of three independent experiments and were analyzed by one-way ANOVA with multiple comparisons, followed by Dunnett post hoc test for significance versus siCTL. $^{**}P<0.01$ and $^{***}P<0.001$ versus siCTL. (**B**) PC-3M cells were transfected as indicated and at 24 hours post-transfection, cells were serum starved for 24 hours, followed by treatment with medium (−) or 100 nM PMA (+) for an additional 24 hours. Whole cell lysates were separated by SDS-PAGE and immunoblotted. (**C**) PC-3M cells were transfected as in A. After a 48-hour culture in medium containing 10% FBS, whole cell lysates were separated by SDS-PAGE and immunoblotted. (**D**) PC-3M cells were transfected, treated with medium (C), 50 ng/ml EGF (E), or 10 ng/ml TNF-α (T), and analyzed as in B. α-Tubulin was used as an internal loading control, and PKD2 and PKD3 were blotted to ensure sufficient knockdown. A quantification of each blot is indicated. Data represent the mean±s.e.m. of three independent experiments. Quantitative data in B and D were analyzed by two-way ANOVA with multiple comparisons, followed by Bonferroni post hoc test for significance. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus siCTL+PMA or siCTL+T. Data in C were analyzed by one-way ANOVA, followed by Dunnett post hoc test for significance versus siCTL. $^{**}P<0.01$ and $^{***}P<0.001$ versus siCTL.

reduced in both cell lines by PKD2 or PKD3 knockdown, although there was little effect on uPA expression. Both EGF and TNF-α did not substantially alter uPAR expression in either cell line, but baseline uPAR expression was significantly downregulated by PKD2 or PKD3 knockdown.

PKD2 and PKD3 contribute to uPA and the gelatinase activity of MMP9

Activation of uPA-uPAR signaling has been shown to activate a cascade of MMPs, subsequently modulating tumor invasion (Dass et al., 2008). To further confirm the effects of PKD2 and PKD3 on uPA and MMP activity, we analyzed uPA activity using a chromogenic approach and assayed the gelatinase activity of MMP9 by zymography. Knockdown of either PKD2 (siPKD2-1, siPKD2-2) or PKD3 (siPKD3-1, siPKD3-2) significantly reduced uPA activity in the culture medium of PC-3M cells; knockdown of both PKD2 and PKD3 (siPKD2+3: siPKD2-1 + siPKD3-1) resulted in an even greater reduction in uPA activity (Fig. 3A, left). Similar results were obtained in DU145 cells in the presence or absence of PMA, and maximal inhibition of uPA activity was obtained with depletion of PKD3 (Fig. 3A, right). Moreover, although no detectable baseline MMP9 activity was observed, PMA-induced MMP9 gelatinase activity was greatly reduced after PKD2 or PKD3 depletion in both DU145 and PC-3M cells (Fig. 3B,C).

Binding of p65 NF-κB to the uPA promoter and subsequent uPA transactivation requires PKD2 and PKD3

Because several studies have demonstrated that NF-κB might have a central role in the transactivation of uPA and uPAR (Killeen et al., 2009; Sliva et al., 2002; Tsunoda et al., 2005), we explored whether PKD2- and/or PKD3-dependent expression and activation of uPA could be attributed to upregulation of NF-κB signaling. As shown in Fig. 4A, the baseline and PMA- (Fig. 4A, left) or TNF-α-induced (Fig. 4A, right) DNA-binding activity of NF-κB to a uPA-specific promoter sequence (uPA-luc) declined dramatically in PC-3M cells in response to PKD2, PKD3 or combined PKD2 and PKD3 depletion. Similarly, the baseline and PMA- (Fig. 4B, left) or TNF-α-induced (Fig. 4B, right) DNA-binding activity of NF-κB to a consensus promoter sequence (2×NF-κB) declined substantially in all PKD-knockdown cells. PMA-induced uPA transactivation was also significantly reduced by PKD2 and/or PKD3 depletion in DU145 cells (Fig. 4C, left). Moreover, silencing of PKD2 or PKD3 with different siRNAs significantly inhibited uPA transactivation in DU145 cells cultured with 10% fetal bovine serum (FBS) (Fig. 4C, right).

To further confirm the possibility that downregulation of uPA transactivation resulting from PKD2 and/or PKD3 depletion was caused by reduced binding of NF-κB to the uPA promoter sequence, we utilized chromatin immunoprecipitation (ChIP). As shown in Fig. 4D, the baseline and PMA-induced binding of p65 NF-κB to the uPA promoter region were dramatically reduced in response to PKD2 or PKD3 depletion. As a negative control, binding of p65 NF-κB to the 11th exon of uPA, which has been shown to not bind p65 NF-κB, was also monitored (Fig. 4D, right); no binding of this negative control over the level of background (which can be attributed to nonspecific binding of the beads) was observed, indicating that the IP and real-time PCR-based amplification of the uPA promoter sequence was

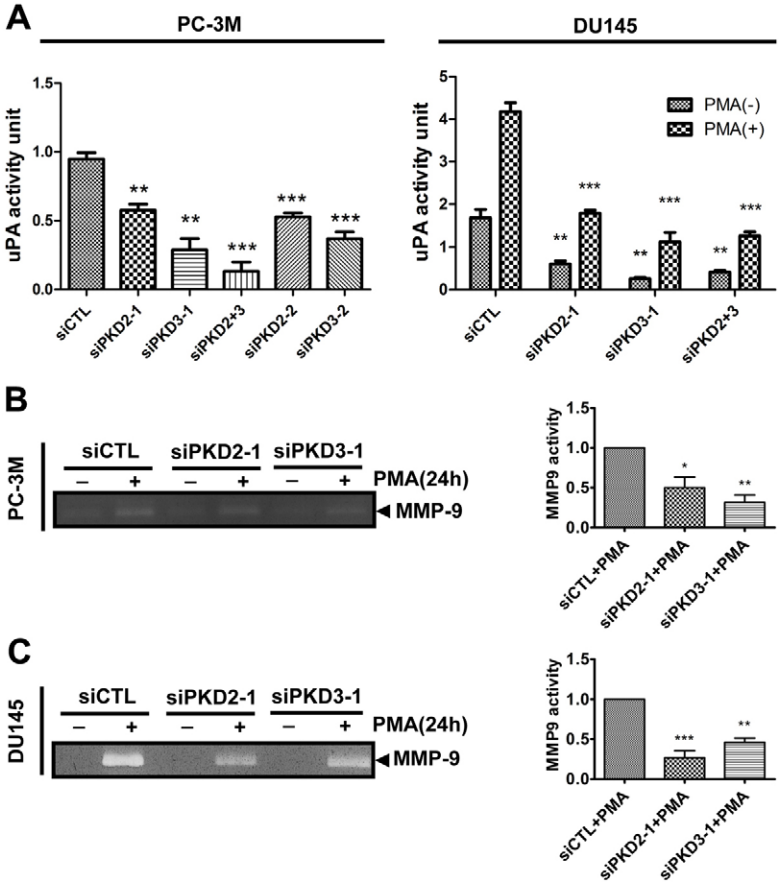

Fig. 3. Depletion of PKD2 and PKD3 impairs uPA and MMP9 activity. (**A**) PC-3M cells (left) were transiently transfected with control siRNA (siCTL), PKD2 siRNA (siPKD2-1 or siPKD2-2), PKD3 siRNA (siPKD3-1 or siPKD3-2), or both siPKD2-1 and siPKD3-1 (siPKD2+3). At 48 hours post-transfection, culture medium was measured for uPA activity (see Materials and Methods). DU145 cells (right) were transfected and analyzed in the same way, except that cells were stimulated with or without PMA (100 nM) for an additional 24 hours after a 24-hour serum starvation. Data represent the mean±s.e.m. of three independent experiments. PC-3M cells (**B**) and DU145 (**C**) were transfected and stimulated as above; the resulting conditioned medium was then concentrated by ultracentrifugation and analyzed by gelatinase zymography. MMP9 bands were normalized to that of the siCTL+PMA sample (shown to the right of the corresponding band). Representative images from three independent experiments are shown. $^{**}P<0.01$ and $^{***}P<0.001$ versus siCTL (with or without PMA as indicated).

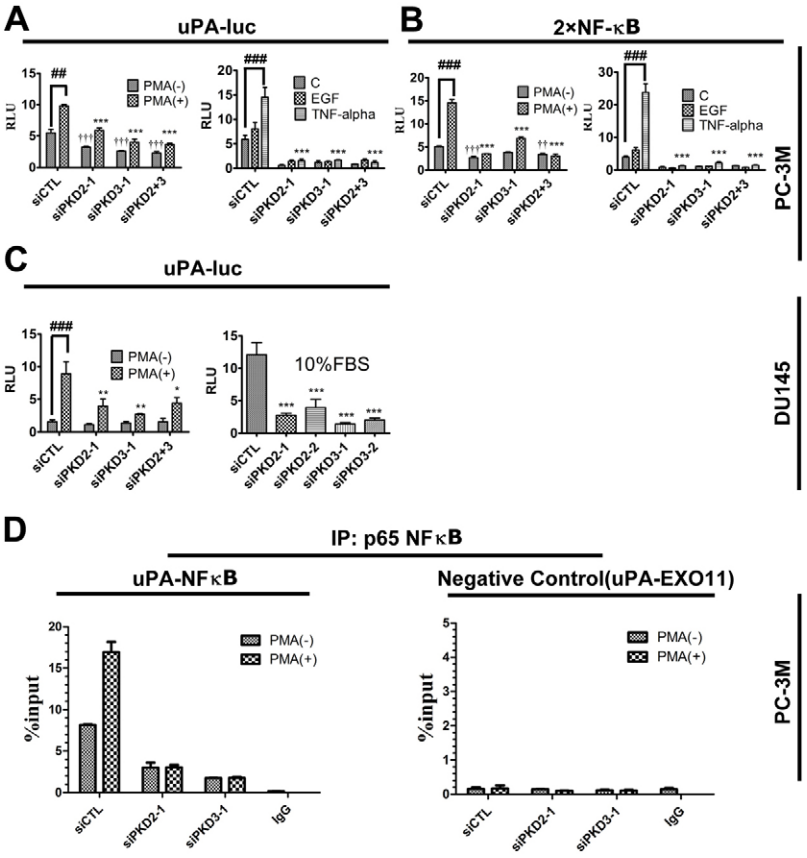

Fig. 4. PKD2 and PKD3 are crucial for binding of p65 to the uPA promoter and subsequent uPA transactivation. (**A**) PC-3M cells were transfected with control siRNA (siCTL), PKD2 siRNA (siPKD2-1), PKD3 siRNA (siPKD3-1), or both (siPKD2+3), combined with a vector containing the uPA promoter sequence driving the firefly luciferase reporter (uPA-luc) in conjunction with a control *Renilla* luciferase expression vector. At 24 hours after transfection, cells were serum starved for an additional 12 hours, followed by 100 nM PMA (left graph), 50 ng/ml EGF (right graph), or 10 ng/ml TNF-α (right graph) treatment for 16 hours. Luciferase reporter activity is presented as the fold activation relative to *Renilla* luciferase activity. (**B**) PC-3M cells were transfected as in A, except that a luciferase reporter vector carrying a double consensus NF-κB binding site (2×NF-κB) was used instead of uPA-luc. (**C**) Left panel: uPA-luc was analyzed in DU145 cells as in A. Right panel: DU145 cells were transfected with siCTL, siPKD2-1 or siPKD2-2, or siPKD3-1 or siPKD3-2, cultured in medium with 10% FBS for 48 h, and analyzed for uPA-luc activity. Data represent the mean ± s.e.m. of three independent experiments and were analyzed by two-way ANOVA with multiple comparisons (PKD-knockdown effect and PMA-treatment effect), followed by Bonferroni post hoc test for significance. ***$P<0.001$ versus siCTL+PMA or TNF-α; $^{\dagger\dagger\dagger}P<0.001$ and $^{\dagger\dagger}P<0.01$ versus siCTL-PMA; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ as labeled. (**D**) PC-3M cells were transfected as indicated, serum starved, and treated as in A. Whole cell lysates were immunoprecipitated with an anti-p65 antibody, co-precipitating chromosome fragments binding to p65 *in vivo* were amplified and quantified by real-time PCR. Results are presented as a ratio of the immunoprecipitated product to the input product. Left panel: real-time PCR of the p65-enriched uPA promoter region. Right panel: real-time PCR of a nonspecific region corresponding to the 11th exon of the uPA gene enriched by p65 (negative control). Data represent the mean ± s.e.m. of two independent experiments.

specific. Taken together, these results indicate that PKD2 and PKD3 have a significant role in NF-κB-mediated transactivation of uPA.

p65 nuclear translocation is primarily dependent on PKD2 rather than PKD3

Although our data suggested that PKD2 and PKD3 were essential for NF-κB-dependent transcription of uPA, the exact mechanisms remained unclear. Because recent studies have reported the occurrence of translocation-independent NF-κB activity, we utilized a subcellular fractionation approach, combined with immunofluorescence, to analyze the influence of PKD2 and PKD3 on the PMA-induced nuclear translocation of p65 NF-κB. As shown in Fig. 5A, PMA-induced p65 NF-κB nuclear translocation was substantially reduced by PKD2 depletion, but only slightly reduced by PKD3 depletion. These differences could not be attributed to different knockdown efficiencies of the PKD2 and PKD3 siRNAs given that both PKD2 and PKD3 protein levels were sufficiently reduced. Consistent with these results, the amount of cytosolic p65 NF-κB decreased significantly upon PMA stimulation in siCTL-transfected cells, but remained constant in PKD2- or PKD3-knockdown cells, both with and without PMA treatment. Additionally, overexpression of PKD2, but not PKD3, caused a significant increase in PMA-induced p65 nuclear translocation in PC-3M cells (Fig. 5A, right).

The role of PKD2 and PKD3 in PMA-induced p65 nuclear distribution was further confirmed by immunofluorescence staining and confocal microscopy (Fig. 5B). As shown in Fig. 5C, PMA treatment induced p65 nuclear translocation in ~78.3% of siCTL-transfected cells, whereas only 6.7%, 43% and 6.7% of cells exhibited a nuclear distribution of p65 in siPKD2-, siPKD3-, and siPKD2+3-transfected cells, respectively. Taken together, these results indicate that PMA-induced p65 nuclear translocation was primarily dependent on PKD2 rather than PKD3.

PKD2 mediates the pIKK-pIκB-Iκ B degradation cascade, and both PKD2 and PKD3 interact with IKKβ

The above results suggest that PKD2 and PKD3, but especially PKD2, have an important role in p65 nuclear translocation. Here, we sought to further elucidate the possible mechanisms underlying p65 nuclear translocation. Because the phosphorylated IκB kinase, phosphorylated inhibitor of NF-κB, IκB (pIKK-pIκB-IκB) degradation cascade is the classical pathway that mediates p65 nuclear translocation, we monitored potential changes in this pathway in response to PKD2 and/or PKD3 knockdown using western blotting. In PC-3M cells, PMA stimulation reduced IKK and IκB phosphorylation to a greater extent in PKD2-knockdown cells than in PKD3-knockdown cells, and greater inhibition of IκB degradation was observed when PKD2 was silenced (Fig. 6A). Similar, albeit weaker, results were obtained when PC-3M cells were treated with TNF-α (Fig. 6B). Collectively, these data suggest that PKD2 is the primary regulator of pIKK-pIκB-mediated IκB degradation in this context. More importantly, we found that endogenous PKD2 or PKD3 co-immunoprecipitated with IKKβ in PC-3M cells, and this interaction could be

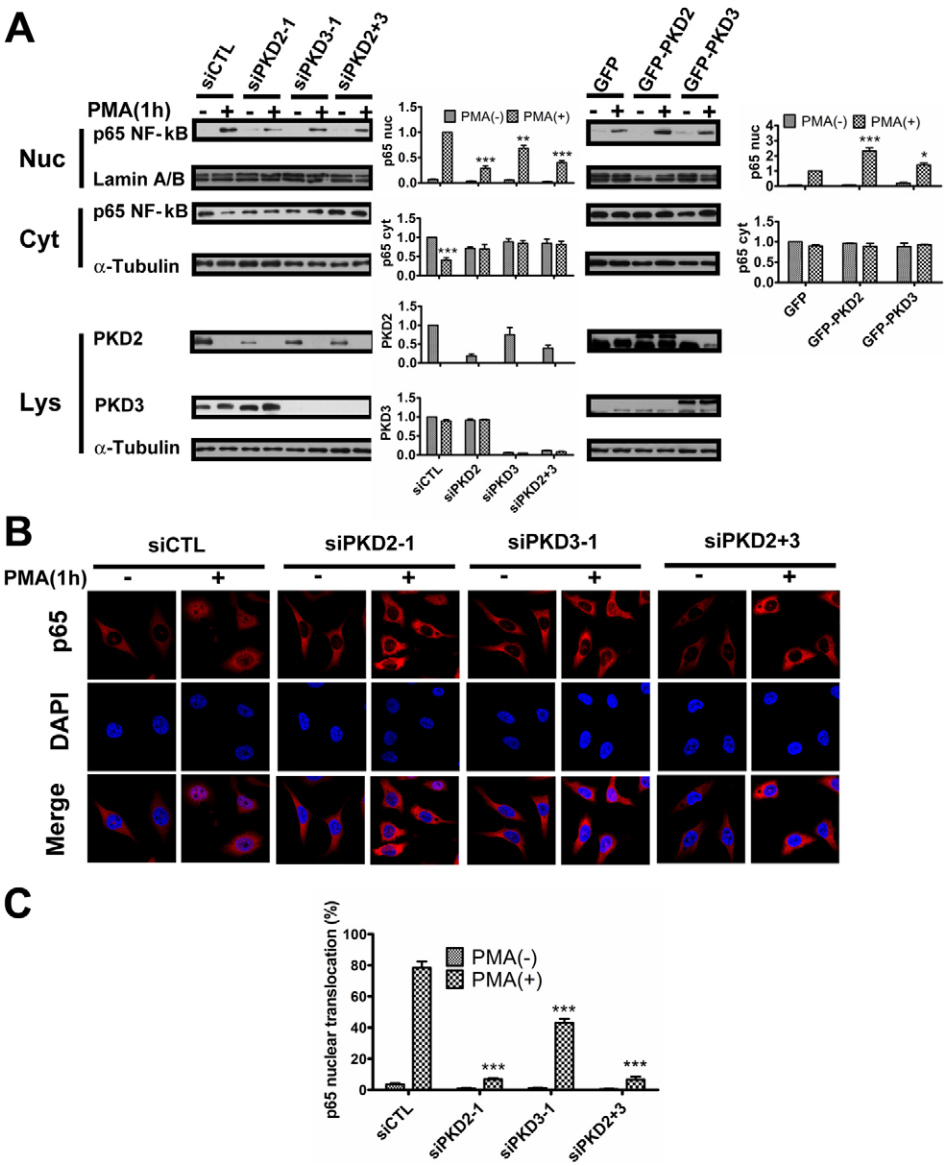

Fig. 5. PKD2 is responsible for p65 NF-κB nuclear translocation. (**A**) Left panel: PC-3M cells were transfected as indicated. At 24 hours post-transfection, cells were serum starved for 24 hours and then left untreated or treated with 100 nM PMA for an additional 1 hour. Levels of nuclear (Nuc) and cytosolic (Cyt) p65 were determined by western blotting. α-Tubulin and lamin A/B were used as internal controls for the cytosolic and nuclear fractions, respectively. PKD2 and PKD3 from whole cell lysates were blotted to ensure sufficient knockdown. Right panel: PC-3M cells were transiently transfected with pEGFP-C2 (GFP), pEGFP-PKD2 (GFP-PKD2) or pEGFP-PKD3 (GFP-PKD3). Cells were treated and proteins were analyzed as described above. PKD2 and PKD3 were blotted from whole cell lysates to ensure overexpression. Data represent the mean±s.e.m. of three independent experiments and were analyzed by two-way ANOVA with multiple comparisons, followed by Bonferroni post hoc test for significance versus control cells. *$P<0.05$; **$P<0.01$; ***$P<0.001$ versus siCTL+PMA. (**B**) Immunofluorescence of p65 localization in PC-3M cells treated as described in A. (**C**) Percentage of cells that exhibited p65 nuclear translocation. Data represent the mean±s.e.m. of three independent experiments and were analyzed by two-way ANOVA with multiple comparisons (PKD-knockdown effect and PMA-treatment effect), followed by Bonferroni post hoc test for significance. ***$P<0.001$ versus siCTL+PMA.

significantly enhanced by PMA stimulation (Fig. 6C). In contrast, PKD2 and PKD3 did not directly interact with p65 with or without PMA treatment (supplementary material Fig. S3).

PKD2 and PKD3 regulate p65 phosphorylation at different sites

In addition to nuclear translocation, phosphorylation at Ser536 or Ser276 of p65 has been reported to be involved in regulating the interaction of p65 with its co-activator CBP/p300 and subsequent transcriptional activation of its target genes (Hu et al., 2004; Jeong et al., 2005; Oeckinghaus and Ghosh, 2009; Zhong et al., 2002). Thus, we evaluated the effects of PKD2 and PKD3 on p65 phosphorylation at Ser276 and Ser536 and subsequent uPA transactivation triggered by PMA and TNF-α. Interestingly, PMA- and TNF-α-induced phosphorylation of p65 at Ser276 was reduced to a greater extent by PKD2 depletion than PKD3 depletion, whereas phosphorylation at Ser536 was reduced mainly by PKD3 silencing in PC-3M cells (Fig. 6A,B).

Rescue of PKD3 depletion phenotypes upon expression of p65 or constitutively active p65

To further confirm that phosphorylation of p65 at Ser536 was involved in PKD3-mediated uPA transactivation, we analyzed whether Ser536 activation could rescue the transcriptional deactivation of uPA caused by PKD3 depletion by co-transfecting a mutant p65 plasmid with PKD3 siRNA. Ser536 of p65 was replaced with either alanine (S536A, dominant-inactive mutant) or aspartic acid (S536D, constitutively active mutant) (Sasaki et al., 2005). As shown in Fig. 7A, overexpression of wild-type p65 (p65WT) or constitutively active p65 (S536D) reversed the downregulation of uPA transactivation observed with PKD3 depletion, and a maximum rescue effect was obtained upon expression of p65 S536D. In contrast, expression of constitutively inactive p65 (S536A) showed little rescue of uPA transactivation.

Next, we investigated whether the reduced tumor cell invasion observed with PKD2 or PKD3 silencing could be rescued by overexpression of p65. As expected, overexpression of p65

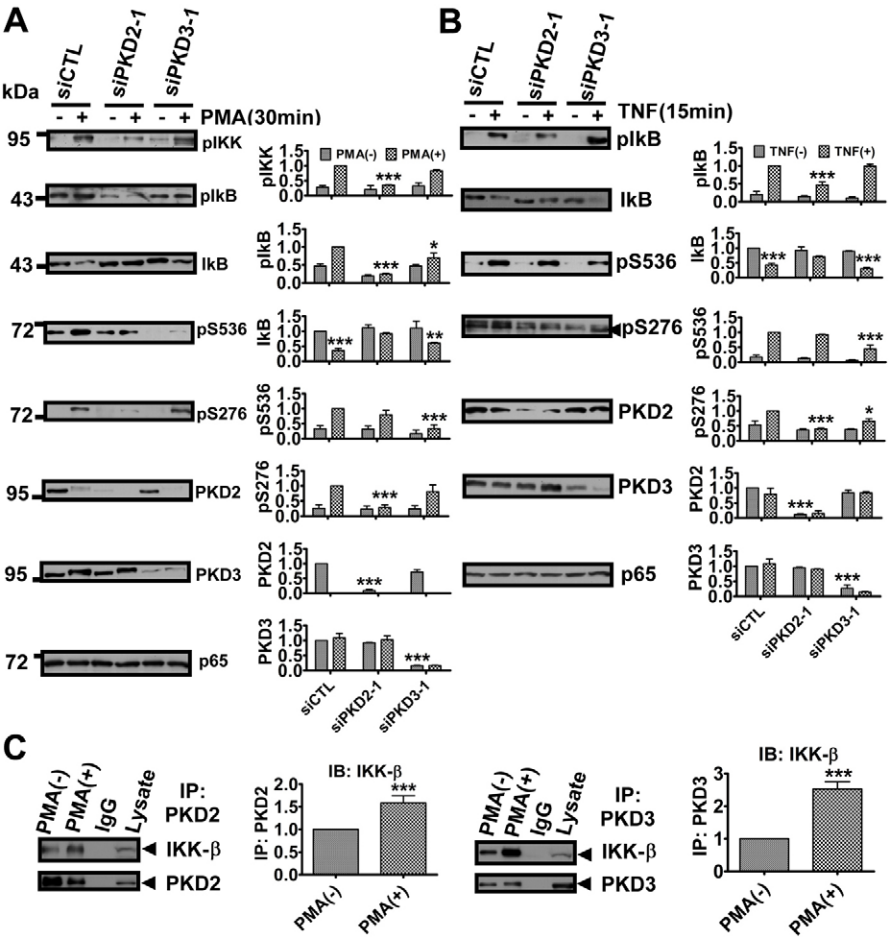

Fig. 6. PKD2 and PKD3 regulate NF-κB nuclear translocation and activation through different pathways. PC-3M cells were transfected as indicated. At 24 hours post-transfection, cells were serum starved for 24 hours and then left untreated or treated with 100 nM PMA for 30 minutes (**A**) or 10 ng/ml TNF-α (**B**) for 15 minutes. Whole cell lysates were separated by SDS-PAGE and assayed with the antibodies against the indicated proteins (pS536 and pS276 indicate p65 phosphorylated on Ser536 or Ser276, respectively). PKD2 and PKD3 expressions were also monitored. Total p65 was determined to ensure equal loading. Data represent the mean ± s.e.m. of three independent experiments and were analyzed by two-way ANOVA with multiple comparisons, followed by Bonferroni post hoc test for significance versus siCTL. $*P<0.05$; $**P<0.01$; $***P<0.001$ versus siCTL+PMA. In the case of IκB, the value for siCTL-transfected cells was set to 1, and $**P<0.01$ and $***P<0.001$ indicate significance for each PMA(+) versus each PMA(−) group. (**C**) PKD2 (left) and PKD3 (right) interact with IKKβ in PC-3M cells. PC-3M cells were grown to 70–80% confluence, serum starved for 24 hours, then stimulated with 100 nM PMA for 15 minutes. Whole cell lysates were immunoprecipitated (IP) with antibodies targeting endogenous PKD2 or PKD3 and co-precipitates with IKKβ were detected by immunoblotting. Data represent the mean±s.e.m. of three independent experiments and were analyzed by Student's *t*-test (2-tailed). $***P<0.001$ versus without PMA.

rescued DU145 cell invasive potential reduced by knockdown of PKD2 or PKD3 (Fig. 7B).

PKD3 interacts with HDAC1, suppresses its expression and decreases its constitutive binding to the uPA promoter

Given that histone deacetylase 1 (HDAC1) binds to the uPA promoter and negatively regulates uPA transcriptional activity in LNCaP cells (Pulukuri et al., 2007), we analyzed the effects of silencing PKD2 or PKD3 on HDAC1 expression in prostate cancer cells. Knockdown of PKD3 significantly enhanced nuclear HDAC1 expression in the absence or presence of PMA stimulation, whereas knockdown of PKD2 upregulated HDAC1 expression only in the presence of PMA treatment (Fig. 8A). As shown in Fig. 8B, PKD3 knockdown enhanced HDAC1 expression at all time points during PMA stimulation, suggesting that PKD3 suppresses the constitutive expression of HDAC1. Likewise, in DU145 cells, depletion of PKD3, much more so than depletion of PKD2, enhanced HDAC1 expression without any stimulation (Fig. 8C). Similarly, overexpression of PKD3 suppressed HDAC1 expression in HEK293 cells (supplementary material Fig. S4). Although the exact mechanism of HDAC1 suppression by PKD3 is not yet clear, PKD3, but not PKD2 (data not shown), constitutively interacted with HDAC1 in PC-3M cells (Fig. 8D).

To further investigate the possibility that PKD3 enhances uPA transactivation through removal of HDAC1 from the uPA promoter, we measured the ability of HDAC1 to bind to the uPA promoter using ChIP followed by real-time PCR. Knockdown of PKD3 enhanced HDAC1 binding to the uPA promoter by ~3.2-fold in the absence of PMA treatment and ~11.8-fold in the presence of PMA. Knockdown of PKD2 did not significantly change HDAC1 binding in the absence of PMA, but amplified it by nearly 2.1-fold in the presence of PMA (Fig. 8E). Although acetylation at Lys9 and/or Lys14 of histone H3, which binds to the uPA promoter, did not change significantly upon PKD3 depletion (supplementary material Fig. S5), we cannot exclude the possibility that PKD3 regulates histone acetylation through HDAC1. Collectively, these data demonstrate that PKD3 has a significant role in the regulation of *HDAC1* gene expression and might activate the uPA promoter by suppressing *HDAC1* expression.

Inhibition of uPA transactivation by PKD3 depletion is rescued by silencing of HDAC1

To test the hypothesis that PKD2 or PKD3 contributes to uPA transactivation by suppressing HDAC1 expression and its subsequent binding to the uPA promoter, we co-transfected HDAC1 and PKD3 siRNAs into PC-3M cells and analyzed uPA transactivation with or without TNF-α stimulation. As expected, knockdown of HDAC1 alone significantly increased baseline uPA transactivation ($P<0.01$ versus siCTL) and this effect was reduced by TNF-α treatment. Moreover, in contrast to single

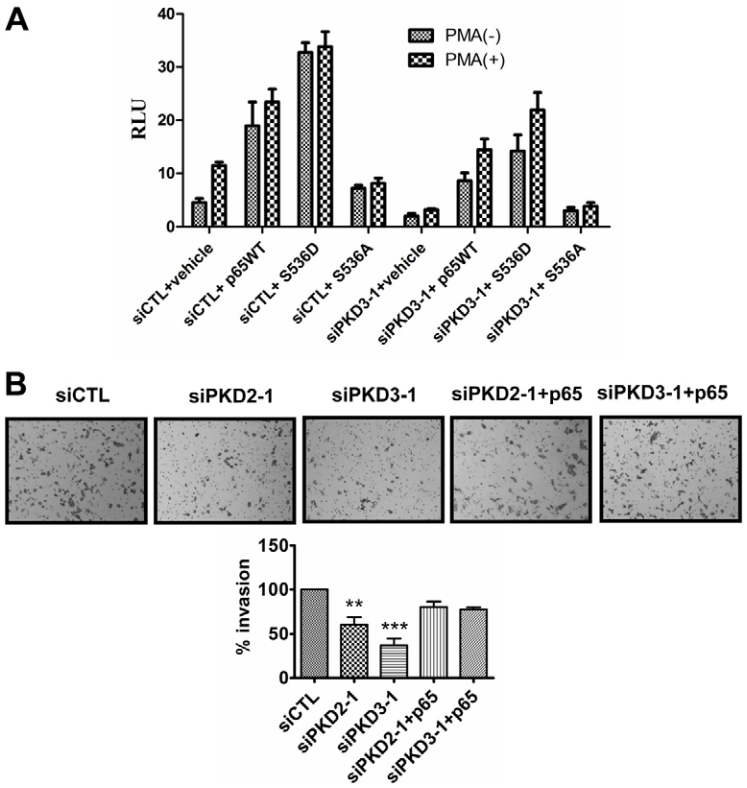

Fig. 7. Rescue of uPA transactivation by forced expression of constitutively active p65 (S536D), and rescue of cell invasion potential by p65 overexpression. (**A**) PKD3 regulates uPA transactivation through p65 phosphorylation at Ser536. PC-3M cells were transfected with control siRNA (siCTL) or PKD3 siRNA (siPKD3-1). At 24 hours after transfection, cells were transfected with vector or plasmids expressing wild-type p65 (p65WT), the constitutively active p65 mutant with the S536D mutation (S536D), or the dominant inactive p65 mutant with S536A mutation (S536A), together with the uPA-luc and *Renilla* reporter plasmids. At 24 hours after the second transfection, cells were serum starved for 12 hours, then treated with medium alone or with 100 nM PMA for 16 hours. Reporter activity is presented as the fold activation relative to *Renilla* luciferase activity. (**B**) p65 overexpression rescued the reduction in DU145 invasion caused by PKD2 and PKD3 silencing. DU145 cells were transfected with siCTL, siPKD2-1 or siPKD3-1. At 24 hours after transfection, cells were transfected with vector or p65WT. At 24 hours after the second transfection, cells were seeded into transwell chambers with or without Matrigel-coated inserts and were cultured for an additional 24 hours. Data were analyzed as in Fig. 1C,D. $^{**}P<0.01$ and $^{***}P<0.001$ versus siCTL.

silencing of PKD3, concomitant depletion of HDAC1 and PKD3 did not significantly decrease uPA transactivation compared with that in the control cells ($P>0.05$; Fig. 8F).

Discussion

In the present study, we showed that both PKD2 and PKD3 contributed to prostate cancer cell invasion by activation of uPA signaling via the NF-κB pathway. Whereas PKD2 served mainly to promote nuclear translocation and Ser276 phosphorylation of p65, PKD3 contributed more to the regulation of Ser536 phosphorylation. Importantly, PKD3 and, to a lesser extent, PKD2 caused reduced expression of nuclear HDAC1 and decreased its binding to the uPA promoter. Conversely, either overexpression of p65 or silencing of HDAC1 could rescue the decrease in uPA transactivation resulting from PKD3 depletion. In summary, PKD2 and PKD3 might play partially overlapping, yet largely distinct, functional roles in prostate cancer cell invasion.

As shown in the current study, double knockdown of PKD2 and PKD3 diminished prostate cancer cell invasion to a greater extent than single knockdown of either protein, indicating that PKD2 and PKD3 have a cooperative role in tumor cell invasion. Moreover, data from RT-qPCR, western blotting, and gelatinase zymography demonstrated that both the expression of uPA signaling proteins, and the activity of uPA and MMP-9 were regulated by PKD2 and PKD3, albeit to different degrees, further implying that PKD2 and PKD3 cooperate to mediate prostate cancer cell invasion through a uPA-uPAR-MMP pathway, which has been reported to be pivotal for prostate cancer invasion (Li and Cozzi, 2007).

Although the PKD1-mediated NF-κB pathway has been shown to regulate cell survival in response to stimuli, such as H_2O_2 and UVB, in a variety of studies (Arun et al., 2011; Song et al., 2009; Storz and Toker, 2003), the current study provides the first evidence that PKD2- and PKD3-mediated NF-κB pathway can promote activation of uPA signaling to regulate prostate cancer cell invasion. PKD2 and PKD3 both appeared to be important for general NF-κB activation and transactivation of uPA by NF-κB. We further investigated the precise mechanisms through which PKD2 and PKD3 mediated NF-κB-dependent uPA transactivation. As expected, PKD2 was primarily responsible for p65 NF-κB nuclear translocation, a process that was only partially blocked by PKD3 depletion. This is plausible because PKD2 is mainly localized within the cytosol, whereas PKD3 shows nuclear distribution; perhaps only cytosolic PKD2 and PKD3 are responsible for the degradation of IκB and subsequent translocation of p65 to the nucleus. Consistent with these results, PKD2 was primarily responsible for IKK phosphorylation and subsequent IκB degradation, indicating that PKD2 has a major role in classical IKK-IκB-mediated NF-κB nuclear translocation. However, whereas the interaction between IKKβ and PKD2 was enhanced by PMA in the current study, elucidation of the mechanism through which PKD2 mediates IKK phosphorylation (directly or indirectly) will require further investigation. Although PKD3 also interacted with IKKβ, and this interaction was dramatically enhanced by PMA treatment, it remains to be determined whether nuclear or cytosolic PKD3 (or both) interacted with IKKβ. Given that PKD3 is primarily localized in the nucleus, the interaction of cytosolic PKD3 with IKKβ might have a similar, but less significant role, to that of PKD2, and the interaction of nuclear PKD3 and IKKβ could play a quite different, but important role(s), within the nucleus. Given that slight inhibition of p65 nuclear translocation does not appear to be sufficient to explain the dramatically decreased transactivation of uPA and subsequent uPA protein expression in response to PKD3 depletion, additional

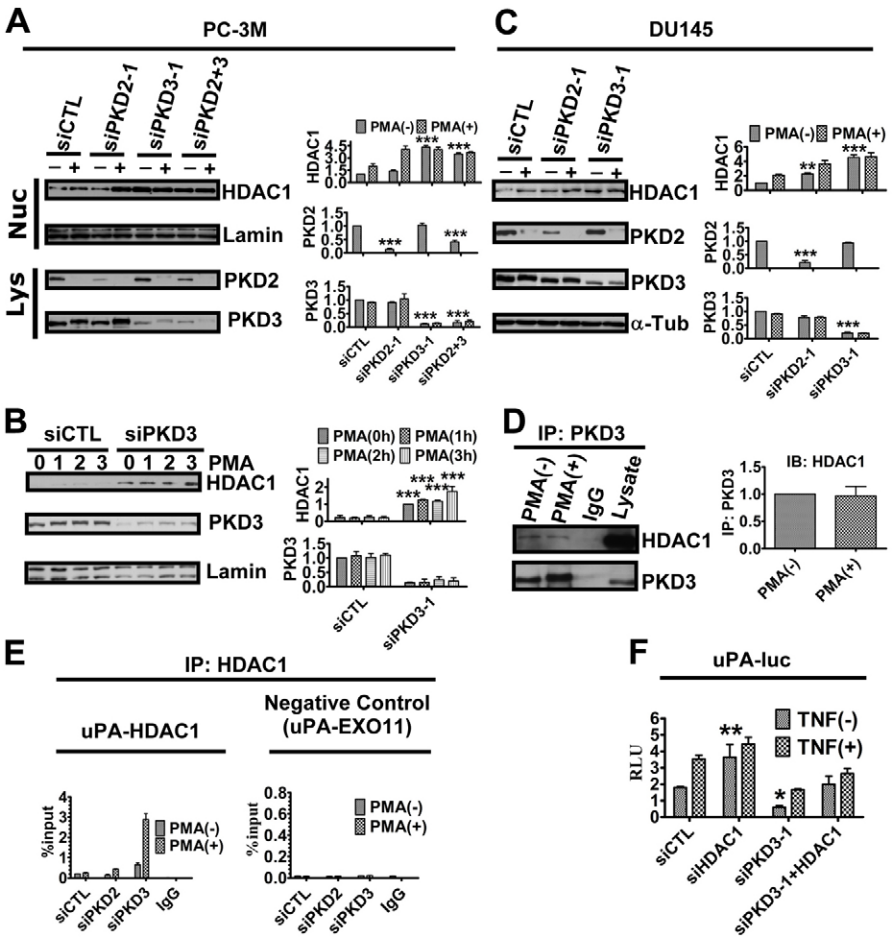

Fig. 8. Nuclear HDAC1 depletion and decreased HDAC1 binding to the uPA promoter are important for uPA transactivation. (**A**) PC-3M cells were transfected as indicated. At 24 hours after transfection, cells were serum-starved for 24 hours followed by treatment with medium alone or with 100 nM PMA for 1 hour. HDAC1 expression in nuclear extracts (Nuc) was determined by western blotting; lamin A/B was used as a loading control. (**B**) PC-3M cells were transfected with siCTL or siPKD3-1. Cells were then serum starved for 24 hours and subsequently treated with 100 nM PMA for the indicated times. HDAC1 expression in whole-cell lysates was assayed by western blotting. (**C**) DU145 cells were transfected as indicated, and HDAC1 expression in whole-cell lysates was assayed. Expression of PKD2 and PKD3 was also measured for A–C. (**D**) PKD3 interacted with HDAC1. PC-3M cells were grown to 70–80% confluence, serum starved for 24 hours, then stimulated with 100 nM PMA for 15 minutes. Whole-cell lysates were immunoprecipitated (IP) with anti-PKD3 antibodies and co-precipitated with HDAC1. Data represent the mean ± s.e.m. of three independent experiments and were analyzed by two-way ANOVA with multiple comparisons, followed by Bonferroni post hoc test for significance versus siCTL. $^{**}P<0.01$ and $^{***}P<0.001$ versus siCTL. (**E**) PKD2 and PKD3 depletion increased HDAC1 binding to the uPA promoter. PC-3M cells were transfected as indicated and treated as in A, then subjected to ChIP and real-time PCR analysis (see Materials and Methods). The ratio of chromatin-precipitated HDAC1 to input HDAC1 is shown as a percentage. Left: real-time PCR of the HDAC1-enriched uPA promoter region. Right: real-time PCR of the nonspecific region corresponding to 11th exon of the uPA gene, enriched by HDAC1. Data represent the mean±s.e.m. of two independent experiments. (**F**) HDAC1 depletion reversed the downregulation of uPA transactivation due to PKD3 knockdown. PC-3M cells were co-transfected with siPKD3-1 or siCTL, and HDAC1 siRNA or siCTL. At 24 hours after transfection, cells were serum starved for 12 hours and treated with 10 ng/ml TNF-α for 16 hours before analysis of reporter activity. Significance was determined by two-way ANOVA. $^{*}P<0.05$ and $^{**}P<0.01$ versus siCTL.

PKD3-mediated mechanisms of uPA transactivation might exist that are independent of p65 translocation.

The current study has also shown that PKD2 was primarily responsible for p65 Ser276 phosphorylation, whereas PKD3 was responsible for p65 Ser536 phosphorylation. Moreover, PKD3-mediated p65 Ser536 phosphorylation is important for the transactivation of uPA, which is strongly supported by the fact that constitutive activation of Ser536 rescues the downregulation of uPA transactivation caused by PKD3 silencing (Fig. 6C). However, Ser276 and/or Ser536 may not be directly phosphorylated by PKD2 or PKD3 given that the amino acid sequences surrounding Ser276 and Ser536 of p65 do not contain a classical PKD substrate motif ([V/I/L]xx[K/R]x[S/T]). Because our previous study has indicated that PKD3 might activate the p38 mitogen-activated protein kinase (MAPK) or phosphoinositol 3-kinase (PI3K)-Akt pathway in prostate cancer cells (Chen et al., 2008), it is plausible that PKD3 might phosphorylate p65 at Ser536 via p38 MAPKs and/or phosphoinositol 3-kinase (PI3K) and Akt. Therefore, the outcome of PKD2- and PKD3-mediated p65 phosphorylation in prostate cancer cell invasion remains to be determined. Most importantly, phosphorylation at Ser536 has been reported to orchestrate p65 activation independently of IκB degradation, because nuclear translocation of phosphorylated p65 (specifically at Ser536), but not total p65, has been shown

to be unaffected by MG-132-dependent inhibition of IκB depletion (Sasaki et al., 2005). Collectively, this suggests that, whereas PKD2 might regulate canonical NF-κB activation through sequential activation of the pIKK-pIκB-IκB degradation p65 translocation cascade and Ser276 phosphorylation, PKD3 may mediate noncanonical NF-κB activation through induction of Ser536 phosphorylation. This could explain why depletion of PKD3 alone dramatically decreased uPA transactivation and expression and reduced the invasive potential of prostate cancer cells, with little effect on p65 nuclear translocation.

Because individual phosphorylation of Ser276 or Ser536 is responsible for transactivation of some, but not all, NF-κB target genes (Dong et al., 2008; Smale, 2010), it is possible that PKD2- or PKD3-dependent p65 phosphorylation at Ser276 and/or Ser536 might play a unique role in the transactivation of different genes (such as those encoding uPA, uPAR, or MT1-MMP).

Interestingly, our evidence that PKD3 suppressed the binding of HDAC1 to the uPA promoter might shed light on our understanding of an unidentified role of PKD3 within the nucleus. HDAC1, a member of the class I HDAC subfamily, has been reported to negatively regulate androgen receptor (AR)-dependent transcription of PSA (Gaughan et al., 2005; Glozak and Seto, 2007; Logan et al., 2006) and to suppress transactivation of NF-κB on the uPA promoter in LNCaP cells. Despite a variety of recent studies demonstrating that nuclear exclusion of class IIa HDACs is triggered by PKD-directed phosphorylation of multiple residues (Jensen et al., 2009; Matthews et al., 2006), the effects of PKDs on HDAC1, especially in the process of prostate cancer invasion, remain unknown. The current study has clearly demonstrated that PKD3 could constitutively interact with HDAC1 and suppress HDAC1 expression. The constitutive nuclear localization of PKD3 in PC-3M and DU145 cells might facilitate its interaction with HDAC1, which is exclusively found in the nucleus, potentially explaining why PKD3 has a greater effect than PKD2 on HDAC1 in untreated cells. Although the exact mechanisms of PKD2- and PKD3-mediated HDAC1 depletion remain to be determined, HDAC1 has been shown to be regulated by ubiquitin-proteasome-mediated degradation in response to multiple kinds of stimuli (TNF-α, IL-1, oxidants, etc.) (Vashisht Gopal and Van Dyke, 2006a; Vashisht Gopal et al., 2006b). Given that IKKβ has been reported to mediate TNF-α-induced HDAC1 degradation, and, in the current study, PKD3 interacted with both IKKβ and HDAC1 in PC-3M cells, PKD3 might act as a functional link between IKKβ and HDAC1, directing HDAC1 ubiquitylation and depletion through IKKβ. Ongoing studies in our laboratory will verify the interaction between PKD3 and HDAC1 *in vitro* and characterize additional partner(s) involved in this potential PKD3–IKKβ–HDAC1 complex. We also cannot exclude the possibility that PKD3 phosphorylates E3 ligases, such as CHFR, thus promoting HDAC1 degradation (Oh et al., 2009).

PKD3 not only suppressed the expression of HDAC1 in whole cell lysates and nuclear fractions, but also decreased the binding of HDAC1 to the uPA promoter region. Moreover, simultaneous silencing of HDAC1 and PKD3 rescued the downregulation of uPA transactivation caused by PKD3 depletion. These results clearly indicate that PKD3 might activate uPA transcription by decreasing HDAC1 protein expression and binding to the uPA promoter. Additionally, PKD3 might be pivotal for shifting the balance of uPA transactivation between NF-κB and HDAC1 at the epigenetic level, thus promoting the invasion and metastasis of prostate cancer cells. Our evidence for a negative role of HDAC1 in prostate cancer invasion is in accordance with that reported by Pulukuri et al. (Pulukuri et al., 2007), but conflicts with reports by Glozak and Seto (Glozak and Seto, 2007) and Kim et al. (Kim et al., 2011), which indicate that HDAC1 might have positive roles in prostate cancer migration and invasion through suppression of E-cadherin expression. However, our evidence demonstrating that PKD3 mediates HDAC1 depletion and the corresponding reduction in HDAC1 binding to the uPA promoter does not exclude the possibility of increased HDAC1 binding to the E-cadherin promoter. Consequently, the use of ChIP-seq technology to monitor alteration of HDAC1 binding to promoter regions of different genes (either tumor-promoting or tumor-repressing) could have predictive value, given that imprudent utilization of HDAC inhibitors to treat cancers might cause unpredicted side effects.

In conclusion, we have demonstrated that PKD2 might serve as a switch to turn on p65 nuclear translocation and activation through Ser276 phosphorylation, and that PKD3 might function to activate p65 via Ser536 phosphorylation while deactivating HDAC1 in highly invasive prostate cancer cells. These processes might all contribute to shifting of the epigenetic balance between NF-κB and HDAC1, initiating transactivation of uPA, and promoting cell invasion. While PKD2 regulates p65 activation through the canonical NF-κB pathway, PKD3 can also trigger its activation in a noncanonical manner (see schematic in supplementary material Fig. S6). Further studies are required to determine whether additional NF-κB targets are also controlled by this cooperative PKD2- and PKD3-mediated mechanism. Nevertheless, the current study provides novel insights into the partially redundant, but largely distinct, roles of different isoforms of the same kinase family in cancer cells.

Materials and Methods

Chemicals and reagents

PMA and all ultrapure reagents were from Promega (Madison, WI, USA) and Sigma (St Louis, MO, USA). uPA activity assay kits were purchased from Millipore (Billerica, MA, USA). Prolong Antifade Reagent was from Molecular Probes (Invitrogen, Carlsbad, CA, USA). Lipofectamine 2000 was from Invitrogen, and HilyMax transfection reagent was from Dojindo (Kamimashikigun, Kumamoto, Japan). The PKD2 Validated Stealth RNAi DuoPak was from Invitrogen (catalog no. 45-3192), and Silencer Selected Validated SiRNA PKD3 was from Ambion (Invitrogen, catalog no. 4390824). BioCoat Matrigel chambers were from BD Biosciences (Franklin Lakes, NJ, USA). The ChIP-IT Express Enzymatic Chromatin Immunoprecipitation Kit was from Active Motif (Carlsbad, CA, USA). All-in-One First-Strand cDNA Synthesis Kit and All-in-One qPCR Mix were from GeneCopoeia (Rockville, MD, USA). Antibodies used for immunoblotting and IP assays were as follows: the HDAC1 primary antibody for chromatin IP was purchased from Millipore; primary antibodies against PKD2 were from Bethyl Laboratories (Montgomery, TX, USA); uPA and MT1-MMP antibodies were from Lab Vision, Thermo Scientific (Kalamazoo, MI, USA); antibodies targeting PKD3, p65, phosphorylated p65 at Ser276 and Ser536, acetylated p65 at Lys310, IKKβ, phosphorylated IKKα/β, IκB, phosphorylated IκB at Ser32 and Ser36, and acetylated histone H3 at Lys9 and Lys14 were purchased from Cell Signaling Technology (Danvers, MA, USA); antibodies targeting uPAR, p65, and all unconjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and Alexa-488- and 594-conjugated secondary antibodies were from Molecular Probes (Invitrogen).

Cell culture, and siRNA and plasmid transfections

PC-3M and DU145 cells were from the American Type Culture Collection and were cultured according to the manufacturer's recommendations. The siRNAs were transfected into cells using Lipofectamine 2000 according to the manufacturer's instructions. When HDAC1 siRNAs were transfected, both strands were pooled together. pEGFP-C2, pEGFP-PKD2, and pEGFP-PKD3 plasmids were transfected into cells using HilyMax according to the

manufacturer's protocol. The following siRNA sequences were used: PKD2-1, 5′-CCUGAGUGUGGCUUCUACGGCCUUU-3′ (Invitrogen validated siRNA, catalog no. 45-3192); PKD2-2, 5′-AAUGACCUUAACUGCCACGUCCCGG-3′ (Azoitei et al., 2010); PKD3-1, 5′-GAACGAGUCUUUGUAGUAAtt-3′ (Silencer Selected Validated siRNA, catalog no. 4390824); PKD3-2, 5′-GAAAGUUCCAC-ACACAUUU-3′ (Chen et al., 2011); HDAC1, 5′-CAGCGACUGUUUGAGAA-CCTT-3′ and 5′-CUAAUGAGCUUCCAUACAATT-3′ (Senese et al., 2007).

Cell migration and invasion assays

For the migration assay, we used confluent cultures (80–90%) in 12-well plates. Twenty-four hours after transfection with the indicated siRNAs, the cell monolayer was scratched with a 10-µl pipette tip, and the length of the cell-free area was monitored at the indicated times using light microscopy. Data are expressed as a percentage of the initial length at time zero. *In vitro* invasion assays were carried out in BD BioCoat Matrigel chambers according to the manufacturer's protocol.

Real-time quantitative RT-PCR amplification

Total RNA from each cell line was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription and quantitative PCR (qPCR) were carried out using the All-in-One First-Strand cDNA Synthesis Kit and All-in-One qPCR Mix (GeneCopoeia) according to the manufacturer's protocol. The following RT-qPCR primers were used: MT1-MMP forward, 5′-CCTTGGACTGTCAGGAATGAG-3′ and reverse, 5′-GAGGGGTCACTGGAA-TGCT-3′; osteopontin forward, 5′-CGCAGACCTGACATCCAGT-3′ and reverse, 5′-GGCTGTCCCAATCAGAAGG-3′; uPA forward, 5′-TGACCCACAGTGGA-AAACAG-3′ and reverse, 5′-TTGTCCTTCAGGGCACATC-3′; uPAR forward, 5′-GGTGACGCCTTCAGCATGA-3′ and reverse, 5′-CCCACTGCGGTACTGG-ACAT-3′; activin A forward, 5′-CGGGTATGTGGAGATAGAGG-3′ and reverse, 5′-GTCTTCCTGGCTGTTCCTGAC-3′; VEGF forward, 5′-TGCAGATTATG-CGGATCAAACC-3′ and reverse, 5′-TGCATTCACATTTGTTGTGCTGTAG-3′; ubiquitin-C (internal control) forward, 5′-ATTTGGGTCGCGGTTCTTG-3′ and reverse, 5′-TGCCTTGACATTCTCGATGGT-3′.

Subcellular fractionation, IP and western blot analysis

Subcellular fractionation was conducted using the Qproteome Cell Compartment Kit following the manufacturer's recommendations. Western blotting and IP were performed as previously described (Lu et al., 2007). Conditioned medium was concentrated using Amicon Ultra-4 Centrifugal Filter Units (Millipore) and was subjected to SDS-PAGE for western blot analysis of secreted proteins.

uPA activity and gelatinase zymography

DU145 and PC-3M cells were transiently transfected with the indicated siRNAs or plasmids, cultured for an additional 24 hours, serum starved for 24 hours, and stimulated with PMA for an additional 24 hours. The resulting conditioned medium was directly assayed using the Chemicon uPA Assay Kit following the manufacturer's protocol or concentrated using Amicon Ultra-4 Centrifugal Filter Units (Millipore) and separated on 10% SDS polyacrylamide gels containing 0.1% (w/v) gelatin under nonreducing conditions. Zymography was then performed as previously described (Cheng et al., 2007).

Indirect immunofluorescence

Cells were seeded on coverslips in 6-well plates and allowed to attach overnight. Immunofluorescent staining was performed as previously described (Lu et al., 2007). Fixed and permeabilized cells were incubated overnight at 4°C with primary antibodies, followed by incubation with Alexa-488- or Alexa 594-conjugated secondary antibodies for 1 hour at room temperature. Finally, coverslips were mounted onto glass slides with Prolong Gold Antifade reagent (after staining the nuclei with DAPI), and stained cells were imaged under a laser scanning confocal fluorescent microscope.

Luciferase reporter assay

PC-3M and DU145 cells were co-transfected with the indicated siRNAs, uPA promoter reporter plasmid (uPA-luc) (Li et al., 2005) and internal control plasmid (pGL4.74[hRluc/TK], from Promega). Twenty-four hours after transfection, the cells were serum starved for 12 hours and subsequently stimulated with PMA (100 nM) for 16 hours. Firefly and *Renilla* luciferase activities of the stimulated cells were measured using the Dual-Luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation assay

ChIP was performed using the ChIP-IT Express Enzymatic Chromatin Immunoprecipitation Kit (Active Motif) with chromatin isolated from 1×10^6 cells, according to the manufacturer's protocol. Quantitative PCR of co-immunoprecipitated genomic DNA fragments was performed with the following promoter-specific primers: uPA-NF-κB sense, 5′-GAGGGGGCGGAAGGGG-AGAA-3′ and uPA-NF-κB antisense, 5′-TGTGGTCAGTTTTGTTTGGATTTG-3′ (Cicek et al., 2009); and nonpromoter sequence primers of the uPA-encoding gene: uPA-EXO11 sense, 5′-TTGTATCTTTGGCGTCACAGG-3′ and uPA-EXO11 antisense, 5′-CATTCTCTTCCTTGGTGTGAC-3′ (Ibañez -Tallon et al., 2002).

Statistical analysis

All statistical analyses were conducted using GraphPad Prism V software. A *P*-value less than 0.05 was considered statistically significant.

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References

Amos, S., Redpath, G. T., Dipierro, C. G., Carpenter, J. E. and Hussaini, I. M. (2010). Epidermal growth factor receptor-mediated regulation of urokinase plasminogen activator expression and glioblastoma invasion via C-SRC/MAPK/AP-1 signaling pathways. *J. Neuropathol. Exp. Neurol.* **69**, 582-592.

Arun, S. N., Kaddour-Djebbar, I., Shapiro, B. A. and Bollag, W. B. (2011). Ultraviolet B irradiation and activation of protein kinase D in primary mouse epidermal keratinocytes. *Oncogene* **30**, 1586-1596.

Azoitei, N., Pusapati, G. V., Kleger, A., Möller, P., Küfer, R., Genze, F., Wagner, M., van Lint, J., Carmeliet, P., Adler, G. et al. (2010). Protein kinase D2 is a crucial regulator of tumour cell-endothelial cell communication in gastrointestinal tumours. *Gut* **59**, 1316-1330.

Chen, C. D. and Sawyers, C. L. (2002). NF-kappa B activates prostate-specific antigen expression and is upregulated in androgen-independent prostate cancer. *Mol. Cell. Biol.* **22**, 2862-2870.

Chen, J., Deng, F., Singh, S. V. and Wang, Q. J. (2008). Protein kinase D3 (PKD3) contributes to prostate cancer cell growth and survival through a PKCepsilon/PKD3 pathway downstream of Akt and ERK 1/2. *Cancer Res.* **68**, 3844-3853.

Chen, J., Shen, Q., Labow, M. and Gaither, L. A. (2011). Protein kinase D3 sensitizes RAF inhibitor RAF265 in melanoma cells by preventing reactivation of MAPK signaling. *Cancer Res.* **71**, 4280-4291.

Chen, L. A., Li, J., Silva, S. R., Jackson, L. N., Zhou, Y., Watanabe, H., Ives, K. L., Hellmich, M. R. and Evers, B. M. (2009). PKD3 is the predominant protein kinase D isoform in mouse exocrine pancreas and promotes hormone-induced amylase secretion. *J. Biol. Chem.* **284**, 2459-2471.

Cheng, J., Huo, D. H., Kuang, D. M., Yang, J., Zheng, L. and Zhuang, S. M. (2007). Human macrophages promote the motility and invasiveness of osteopontin-knock-down tumor cells. *Cancer Res.* **67**, 5141-5147.

Cicek, M., Fukuyama, R., Cicek, M. S., Sizemore, S., Welch, D. R., Sizemore, N. and Casey, G. (2009). BRMS1 contributes to the negative regulation of uPA gene expression through recruitment of HDAC1 to the NF-kappaB binding site of the uPA promoter. *Clin. Exp. Metastasis* **26**, 229-237.

Dass, K., Ahmad, A., Azmi, A. S., Sarkar, S. H. and Sarkar, F. H. (2008). Evolving role of uPA/uPAR system in human cancers. *Cancer Treat. Rev.* **34**, 122-136.

Dong, J., Jimi, E., Zhong, H., Hayden, M. S. and Ghosh, S. (2008). Repression of gene expression by unphosphorylated NF-kappaB p65 through epigenetic mechanisms. *Genes Dev.* **22**, 1159-1173.

Eiseler, T., Döppler, H., Yan, I. K., Goodison, S. and Storz, P. (2009). Protein kinase D1 regulates matrix metalloproteinase expression and inhibits breast cancer cell invasion. *Breast Cancer Res.* **11**, R13.

Eiseler, T., Hausser, A., De Kimpe, L., Van Lint, J. and Pfizenmaier, K. (2010). Protein kinase D controls actin polymerization and cell motility through phosphorylation of cortactin. *J. Biol. Chem.* **285**, 18672-18683.

Festuccia, C., Angelucci, A., Gravina, G. L., Biordi, L., Millimaggi, D., Muzi, P., Vicentini, C. and Bologna, M. (2005). Epidermal growth factor modulates prostate cancer cell invasiveness regulating urokinase-type plasminogen activator activity. EGF-receptor inhibition may prevent tumor cell dissemination. *Thromb. Haemost.* **93**, 964-975.

Gaughan, L., Logan, I. R., Neal, D. E. and Robson, C. N. (2005). Regulation of androgen receptor and histone deacetylase 1 by Mdm2-mediated ubiquitylation. *Nucleic Acids Res.* **33**, 13-26.

Glozak, M. A. and Seto, E. (2007). Histone deacetylases and cancer. *Oncogene* **26**, 5420-5432.

Guerrini, L., Casalino, L., Corti, A. and Blasi, F. (1996). NF-kappa B-mediated regulation of urokinase gene expression by PMA and TNF-alpha in human A549 cells. *FEBS Lett.* **393**, 69-73.

Hausser, A., Storz, P., Märtens, S., Link, G., Toker, A. and Pfizenmaier, K. (2005). Protein kinase D regulates vesicular transport by phosphorylating and activating phosphatidylinositol-4 kinase IIIbeta at the Golgi complex. *Nat. Cell Biol.* **7**, 880-886.

Hu, J., Nakano, H., Sakurai, H. and Colburn, N. H. (2004). Insufficient p65 phosphorylation at S536 specifically contributes to the lack of NF-kappaB activation and transformation in resistant JB6 cells. *Carcinogenesis* **25**, 1991-2003.

Huang, S., Pettaway, C. A., Uehara, H., Bucana, C. D. and Fidler, I. J. (2001). Blockade of NF-kappaB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis. *Oncogene* **20**, 4188-4197.

Ibañez-Tallon, I., Ferrai, C., Longobardi, E., Facetti, I., Blasi, F. and Crippa, M. P. (2002). Binding of Sp1 to the proximal promoter links constitutive expression of the human uPA gene and invasive potential of PC3 cells. *Blood* **100**, 3325-3332.

Jadali, A. and Ghazizadeh, S. (2010). Protein kinase D is implicated in the reversible commitment to differentiation in primary cultures of mouse keratinocytes. *J. Biol. Chem.* **285**, 23387-23397.

Jaggi, M., Rao, P. S., Smith, D. J., Wheelock, M. J., Johnson, K. R., Hemstreet, G. P. and Balaji, K. C. (2005). E-cadherin phosphorylation by protein kinase D1/protein kinase Cmu is associated with altered cellular aggregation and motility in prostate cancer. *Cancer Res.* **65**, 483-492.

Jarrard, D. F., Blitz, B. F., Smith, R. C., Patai, B. L. and Rukstalis, D. B. (1994). Effect of epidermal growth factor on prostate cancer cell line PC3 growth and invasion. *Prostate* **24**, 46-53.

Jensen, E. D., Gopalakrishnan, R. and Westendorf, J. J. (2009). Bone morphogenic protein 2 activates protein kinase D to regulate histone deacetylase 7 localization and repression of Runx2. *J. Biol. Chem.* **284**, 2225-2234.

Jeong, S. J., Pise-Masison, C. A., Radonovich, M. F., Park, H. U. and Brady, J. N. (2005). A novel NF-kappaB pathway involving IKKbeta and p65/RelA Ser-536 phosphorylation results in p53 Inhibition in the absence of NF-kappaB transcriptional activity. *J. Biol. Chem.* **280**, 10326-10332.

Killeen, S. D., Wang, J. H., Andrews, E. J. and Redmond, H. P. (2009). Bacterial endotoxin enhances colorectal cancer cell adhesion and invasion through TLR-4 and NF-kappaB-dependent activation of the urokinase plasminogen activator system. *Br. J. Cancer* **100**, 1589-1602.

Kim, M. J., Kim, D. H., Na, H. K. and Surh, Y. J. (2010a). TNF-α induces expression of urokinase-type plasminogen activator and β-catenin activation through generation of ROS in human breast epithelial cells. *Biochem. Pharmacol.* **80**, 2092-2100.

Kim, Y. I., Park, J. E., Brand, D. D., Fitzpatrick, E. A. and Yi, A. K. (2010b). Protein kinase D1 is essential for the proinflammatory response induced by hypersensitivity pneumonitis-causing thermophilic actinomycetes Saccharopolyspora rectivirgula. *J. Immunol.* **184**, 3145-3156.

Kim, N. H., Kim, S. N. and Kim, Y. K. (2011). Involvement of HDAC1 in E-cadherin expression in prostate cancer cells; its implication for cell motility and invasion. *Biochem. Biophys. Res. Commun.* **404**, 915-921.

Kleger, A., Loebnitz, C., Pusapati, G. V., Armacki, M., Müller, M., Tümpel, S., Illing, A., Hartmann, D., Brunner, C., Liebau, S. et al. (2011). Protein kinase D2 is an essential regulator of murine myoblast differentiation. *PLoS ONE* **6**, e14599.

Li, H., Ye, X., Mahanivong, C., Bian, D., Chun, J. and Huang, S. (2005). Signaling mechanisms responsible for lysophosphatidic acid-induced urokinase plasminogen activator expression in ovarian cancer cells. *J. Biol. Chem.* **280**, 10564-10571.

Li, Y. and Cozzi, P. J. (2007). Targeting uPA/uPAR in prostate cancer. *Cancer Treat. Rev.* **33**, 521-527.

Logan, I. R., Gaughan, L., McCracken, S. R., Sapountzi, V., Leung, H. Y. and Robson, C. N. (2006). Human PIRH2 enhances androgen receptor signaling through inhibition of histone deacetylase 1 and is overexpressed in prostate cancer. *Mol. Cell. Biol.* **26**, 6502-6510.

Lu, G., Chen, J., Espinoza, L. A., Garfield, S., Toshiyuki, S., Akiko, H., Huppler, A. and Wang, Q. J. (2007). Protein kinase D 3 is localized in vesicular structures and interacts with vesicle-associated membrane protein 2. *Cell. Signal.* **19**, 867-879.

Matthews, S. A., Liu, P., Spitaler, M., Olson, E. N., McKinsey, T. A., Cantrell, D. A. and Scharenberg, A. M. (2006). Essential role for protein kinase D family kinases in the regulation of class II histone deacetylases in B lymphocytes. *Mol. Cell. Biol.* **26**, 1569-1577.

McEneaney, V., Dooley, R., Harvey, B. J. and Thomas, W. (2010). Protein kinase D stabilizes aldosterone-induced ERK1/2 MAP kinase activation in M1 renal cortical collecting duct cells to promote cell proliferation. *J. Steroid Biochem. Mol. Biol.* **118**, 18-28.

Oeckinghaus, A. and Ghosh, S. (2009). The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harb. Perspect. Biol.* **1**, a000034.

Oh, Y. M., Kwon, Y. E., Kim, J. M., Bae, S. J., Lee, B. K., Yoo, S. J., Chung, C. H., Deshaies, R. J. and Seol, J. H. (2009). Chfr is linked to tumour metastasis through the downregulation of HDAC1. *Nat. Cell Biol.* **11**, 295-302.

Palayoor, S. T., Youmell, M. Y., Calderwood, S. K., Coleman, C. N. and Price, B. D. (1999). Constitutive activation of IkappaB kinase alpha and NF-kappaB in prostate cancer cells is inhibited by ibuprofen. *Oncogene* **18**, 7389-7394.

Papazyan, R., Doche, M., Waldron, R. T., Rozengurt, E., Moyer, M. P. and Rey, O. (2008). Protein kinase D isozymes activation and localization during mitosis. *Exp. Cell Res.* **314**, 3057-3068.

Park, J. E., Kim, Y. I. and Yi, A. K. (2009). Protein kinase D1 is essential for MyD88-dependent TLR signaling pathway. *J. Immunol.* **182**, 6316-6327.

Peterburs, P., Heering, J., Link, G., Pfizenmaier, K., Olayioye, M. A. and Hausser, A. (2009). Protein kinase D regulates cell migration by direct phosphorylation of the cofilin phosphatase slingshot 1 like. *Cancer Res.* **69**, 5634-5638.

Pulukuri, S. M., Gorantla, B. and Rao, J. S. (2007). Inhibition of histone deacetylase activity promotes invasion of human cancer cells through activation of urokinase plasminogen activator. *J. Biol. Chem.* **282**, 35594-35603.

Sasaki, C. Y., Barberi, T. J., Ghosh, P. and Longo, D. L. (2005). Phosphorylation of RelA/p65 on serine 536 defines an IkappaBalpha-independent NF-kappaB pathway. *J. Biol. Chem.* **280**, 34538-34547.

Senese, S., Zaragoza, K., Minardi, S., Muradore, I., Ronzoni, S., Passafaro, A., Bernard, L., Draetta, G. F., Alcalay, M., Seiser, C. et al. (2007). Role for histone deacetylase 1 in human tumor cell proliferation. *Mol. Cell. Biol.* **27**, 4784-4795.

Sliva, D., English, D., Lyons, D. and Lloyd, F. P., Jr (2002). Protein kinase C induces motility of breast cancers by upregulating secretion of urokinase-type plasminogen activator through activation of AP-1 and NF-kappaB. *Biochem. Biophys. Res. Commun.* **290**, 552-557.

Smale, S. T. (2010). Selective transcription in response to an inflammatory stimulus. *Cell* **140**, 833-844.

Song, J., Li, J., Qiao, J., Jain, S., Mark Evers, B. and Chung, D. H. (2009). PKD prevents H2O2-induced apoptosis via NF-kappaB and p38 MAPK in RIE-1 cells. *Biochem. Biophys. Res. Commun.* **378**, 610-614.

Stock, D., Groome, P. A. and Siemens, D. R. (2008). Inflammation and prostate cancer: a future target for prevention and therapy? *Urol. Clin. North Am.* **35**, 117-130.

Storz, P. and Toker, A. (2003). Protein kinase D mediates a stress-induced NF-kappaB activation and survival pathway. *EMBO J.* **22**, 109-120.

Syed, V., Mak, P., Du, C. and Balaji, K. C. (2008). Beta-catenin mediates alteration in cell proliferation, motility and invasion of prostate cancer cells by differential expression of E-cadherin and protein kinase D1. *J. Cell. Biochem.* **104**, 82-95.

Tsunoda, K., Kitange, G., Anda, T., Shabani, H. K., Kaminogo, M., Shibata, S. and Nagata, I. (2005). Expression of the constitutively activated RelA/NF-kappaB in human astrocytic tumors and the in vitro implication in the regulation of urokinase-type plasminogen activator, migration, and invasion. *Brain Tumor Pathol.* **22**, 79-87.

Vashisht Gopal, Y. N. and Van Dyke, M. W. (2006a). Depletion of histone deacetylase protein: a common consequence of inflammatory cytokine signaling? *Cell Cycle* **5**, 2738-2743.

Vashisht Gopal, Y. N., Arora, T. S. and Van Dyke, M. W. (2006b). Tumour necrosis factor-alpha depletes histone deacetylase 1 protein through IKK2. *EMBO Rep.* **7**, 291-296.

Wang, Q. J. (2006). PKD at the crossroads of DAG and PKC signaling. *Trends Pharmacol. Sci.* **27**, 317-323.

Zhong, H., May, M. J., Jimi, E. and Ghosh, S. (2002). The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. *Mol. Cell* **9**, 625-636.