AWARD NUMBER: W81XWH-16-1-0173

TITLE: Targeting a Novel Androgen Receptor-Repressed Pathway in Prostate Cancer Therapy

PRINCIPAL INVESTIGATOR: Qiming J. Wang, PhD

CONTRACTING ORGANIZATION: University of Pittsburgh

REPORT DATE: SEPTEMBER 2019

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
SEPTEMBER 2019	Annual	1 Sep 2018 - 31 Aug 2019

4. TITLE AND SUBTITLE

Targeting a Novel Androgen Receptor-Repressed Pathway in Prostate Cancer Therapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-1-0173

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Pittsburgh

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The central hypothesis of the proposal is that PKD plays a crucial role in limiting the effectiveness of ADT by increasing prostate cancer survival through upregulating AURKA expression. AURKA encodes the protein Aurora A kinase, which is a master cell cycle regulator that is often dysregulated in human cancers. Our data demonstrated an important role of PKD in regulating Aurora A stability, implying that PKD's role in cancer cell survival and proliferation may be mediated through Aurora A. The primary goal of this study is to investigate the potential roles of PKD in mediating therapeutic resistance to ADT and to investigate the impact of PKD SMI-based combination therapies to curtail ADT-induced therapy resistance. This remains our main focus during the third funding cycle. Major efforts were devoted to Aim 3, where we seek to determine the functional input of PKD in ADT resistance in vivo and assess the efficacy of PKD SMI in combination with AR antagonists in prostate cancer mouse models. The work is still on-going since the animal studies take longer time to complete. We have two manuscripts under preparation. A 6-month extension of this project has been approved.

15. SUBJECT TERMS

Prostate cancer, protein kinase D, androgen, androgen receptor, therapeutic resistance, androgen-deprivation therapy, Aurora A, cell cycle.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	13	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. INTRODUCTION:

Prostate cancer progression to castration resistance associates with poor prognosis and high mortality. Androgen deprivation therapy (ADT) has been the standard therapy for advanced metastatic prostate cancer. However, ADT has been linked to the development of therapy resistance in part through inducing prosurvival adaptive responses. Increased understandings of these adaptive mechanisms will lead to discovery of new targets/therapies for mCRPC. We will investigate the roles of protein kinase D (PKD) in therapeutic resistance to ADT and the impact of PKD inhibitor-based combination therapies to curtail ADT-induced therapy resistance.

2. KEYWORDS:

Prostate cancer, androgen, androgen receptor, Protein kinase D, androgen-deprivation therapy, therapy resistance, transcriptional regulation, aurora A kinase, mitosis, cell cycle.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

We seek to test **the central hypothesis is that PKD plays a crucial role in limiting the effectiveness of ADT by increasing prostate cancer survival through upregulating AURKA expression, and PKD small molecule inhibitors may enhance the efficacy of AR antagonists in prostate cancer treatment.** Our study will provide insights to the role of PKD in ADT-induced prosurvival responses relevant to the progression to CRPC. Successful completion of this study will define the role and mechanisms of PKD in treatment (ADT)-induced prostate cancer resistance. Three Specific Aims are proposed: **Aim 1.** Determine the mechanisms through which AR represses PKD1 expression in androgen-sensitive prostate cancer cells. **Aim 2.** Test the hypothesis that androgen deprivation-induced PKD1 expression promotes prostate cancer cell survival and ADT resistance through upregulating AURKA and CENPE. **Aim 3.** Determine the functional input of PKD in ADT resistance *in vivo* and assess the efficacy of PKD SMI in combination with AR antagonists in prostate cancer tumor xenografts.

What was accomplished under these goals?

MAJOR ACTIVITIES

The primary goal of this study is to investigate the potential roles of PKD in mediating therapeutic resistance to ADT and to investigate the impact of PKD SMI-based combination therapies to curtail ADT-induced therapy resistance. This remains our main focus during the third funding cycle. Major efforts were devoted to Aim 3, where we seek to determine the functional input of PKD in ADT resistance *in vivo* and assess the efficacy of PKD SMI in combination with AR antagonists in prostate cancer mouse models. The work is still on-going. A 6-month extension of this project has been approved.

SPECIFIC OBJECTIVES

Here is a list of major tasks and milestones to be achieved:

Specific Aim 1. Determine the mechanisms through which AR represses PKD1 expression in androgen-

sensitive prostate cancer cells. [This aim has been completed]

Major Task 1: Test the hypothesis that FRS2 is required for the repression of PKD1 by AR.

Major Task 2: Determine the downstream targets of FRS2 that mediate PKD1 repression by androgen

Specific Aim 2. Test the hypothesis that androgen deprivation-induced PKD1 expression promotes prostate cancer cell survival and ADT resistance through upregulating AURKA and CENPE. [Study has been largely completely]

Major Task 3: Test the hypothesis that androgen deprivation-induced PKD1 expression.

Major Task 4: Test the hypothesis that PKD promotes resistance to antiandrogens through modulating Aurora-A and Cenp-E expression and mitotic programing.

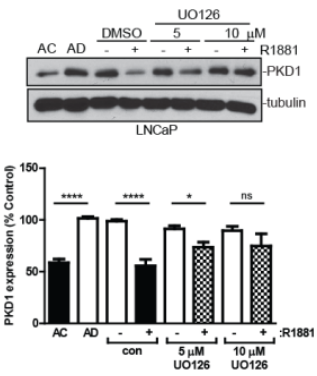
Specific Aim 3. Determine the functional input of PKD in ADT resistance *in vivo* and assess the efficacy of PKD SMI in combination with AR antagonists in prostate cancer tumor xenografts. [Study is on-going]

Major Task 5: Determine that increased PKD1 expression confers resistance to AR antagonists in PrCa xenograft models.

Major Task 6: Determine that targeted inhibition of PKD by CRT101 enhances the efficacy of AR antagonists *in vitro* and *in vivo*.

SIGNIFICANT RESULTS (The progress is outlined along with the statement of work)

Specific Aim 1 Determine the mechanisms through which AR represses PKD1 expression in androgen-sensitive prostate cancer cells.	**Timeline and completion status**
Major Task 1: Test the hypothesis that FRS2 is required for the repression of PKD1 by AR **Major goals have been accomplished. The work has been published (*Zhang et al., Oncotarget, 2017, 8:12800-12811*) (See progress report for YR 2016-2017).**	
Subtask 1: Determine the mRNA and protein levels of FGFs, FGF-BP, FGFR1-4, FRS2, and PKD1, and phosphorylation of FRS2 in prostate cancer cells upon androgen depletion +/- R1881. This subtask has been investigated but not completed.	1-4 50% completed
Subtask 2: Determine the specific FGF2, FGFR, and phosphorylation of FRS2 required for androgen-induced PKD1 repression. This subtask has been partially completed. Our data have demonstrated the essential role of FGF2/FGFR and FRS2 for androgen-induced PKD1 repression. We have detected FGF2, FGFR and phosphorylation of FRS2 in prostate cancer cells. Their relevance to PKD1 suppression by androgen remains to be further exploited.	70% completed
Subtask 3: Determine if increased FGF2 is due to liberation of entrapped FGF-2 from extracellular matrix through activation of heparinase, which leads to activation of FGFR	1-3

and downregulation of PKD. This subtask is on-going.	on-going
Milestone(s) Achieved: Identification of specific FGF2, FGFR, and phosphorylation sites of FRS2 involved in the repression of PKD1 by androgen. Our data demonstrated an important role of FGF2, FGFR, and phosphorylation of FRS2 in the repression of PKD1 by androgen. The detailed molecular mechanisms remain to be further defined. Thus, the milestones are partially achieved.	70% completed
Major Task 2: Determine the downstream targets of FRS2 that mediate PKD1 repression by androgen **This task has been partially completed. The findings were described in Zhang et al., Oncotarget, 2017, 8:12800-12811. (See progress report for YR 2016-2017).**	
Subtask 1: Determine the activity of MEK/ERK, PI3K/Akt, and PLCγ/PKC pathways upon androgen depletion +/- R1881in LNCaP cells. We have identified MEK/ERK as a major mediator for the suppression of PKD1 by androgen.	completed
Subtask 2: Using selective inhibitors and siRNAs targeting the MEK/ERK, PI3K/Akt, and PLCγ/PKC pathways to determine their contributions to PKD1 expression upon androgen depletion. We have used the inhibitors of the MEK/ERK, PI3K/Akt, and PLCγ/PKC pathways. Our data indicated that MEK/ERK is the major mediator of the effect of androgen on PKD1 expression. The results on MEK inhibitor UO126 are described in our Oncotarget paper.  Fig. 1. Androgen repression of PKD1 is dependent on the MEK/ERK signaling pathway. LNCaP cells were grown in AC or AD medium for 48 h, followed by treatment with or without R1881 in the presence or absence of the MEK inhibitor UO0126 for 16 h. Cell lysates were subjected to immunoblotting for PKD1. Representative images from one of at least three independent experiments are shown. bottom, quantitative measurement of band intensity by densitometry analysis. Data are the Mean ± SEM of five to seven independent experiments.	completed
Subtask 3: Conduct detail analysis of the pathway identified in Subtask 2 and assessing its impact on PKD1 expression. The effect of this pathway on PKD1 expression has been characterized and described in our Oncotarget paper.	completed

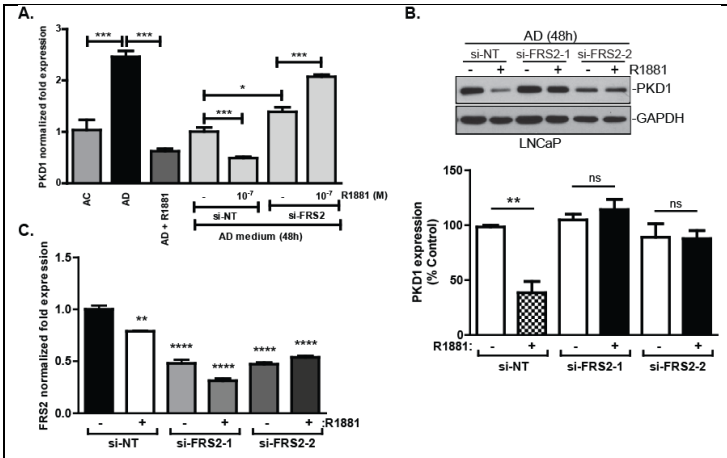


Figure 2. FRS2 was required for androgen-induced PKD1 repression. A. Knockdown of FRS2 reversed the repression of PKD1 transcription by androgen. Cells were transfected with a FRS2 siRNA (si-FRS2) and a non-targeting siRNA (si-NT), followed by treatment with or without R1881. PKD1 transcripts were analyzed by real time RT-qPCR. Representative data from one of three independent experiments with triplicate measurements are shown. **B. Knockdown of FRS2 blocked the repression of PKD1 protein by androgen.** LNCaP cells were transfect with two different FRS2 siRNAs (si-FRS2-1, -2), followed by treatment with R1881. *right*, quantitative measurement of band intensity for PKD1 from three experiments is shown. **C. Real time RT-PCR confirmed the knockdown of FRS2.** Cells from "B" were subjected to RNA extraction, followed by real time RT-PCR for levels of PKD1 transcript.

Milestone(s) Achieved: Identification of the specific pathway involved in the regulation of PKD1 expression by androgen. We have achieved this milestone by identify MEK/ERK as the major signaling pathway mediating the effect of androgen on PKD1.	completed
Specific Aim 2 Test the hypothesis that androgen deprivation-induced PKD1 expression promotes prostate cancer cell survival and ADT resistance through upregulating AURKA and CENPE.	
Major Task 3: Test the hypothesis that androgen deprivation-induced PKD1 expression promotes tumor cell survival and confers resistance to AR antagonists in PrCa cells. **Please see description under each subtask for the progress of the studies.**	
Subtask 1: Determine the effects of knockdown of PKD1 by siRNAs on sensitivity of AR antagonists, bicalutamide and enzalutamide, in androgen-sensitivity PrCa cells. The effect of knockdown of PKD1 on AR antagonist sensitivity has been evaluated in PrCa cells. Our data showed that knockdown of PKD sensitized PrCa cells to enzalutamide-induced cell death. A manuscript describing these findings is under preparation.	Completed

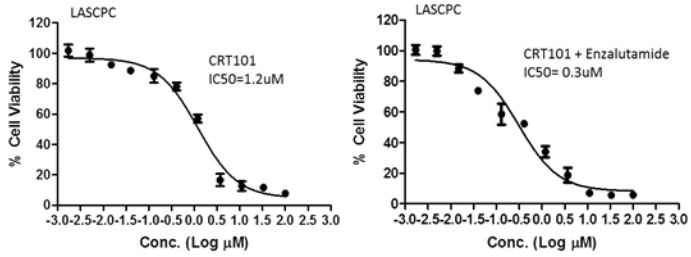
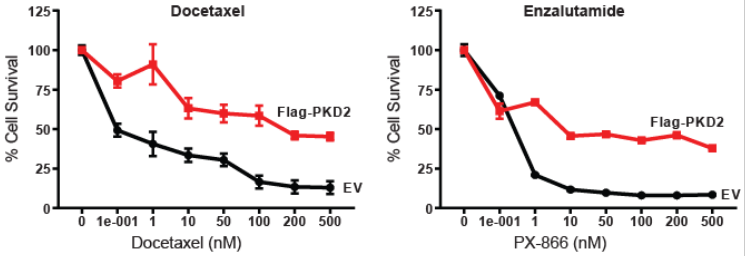


Figure 3. Loss of PKD activity sensitized LASCPC prostate cancer cells to enzalutamide. Cells were treated with increasing concentrations of PKD inhibitor CRT101 and CRT101 + enzalutamide. Cell viability was measured by CCK8 assay after 72 hours. The data is the mean ± SEM of three independent experiments.

Subtask 2: Determine the effects of PKD1 overexpression on sensitivity of AR antagonists, bicalutamide and enzalutamide, in androgen-sensitivity PrCa cells. This subtask has been completed and the results are described in YR 2016-2017 progress report. A manuscript describing these findings is under preparation.	completed
Milestone(s) Achieved: Obtain the data that support the concept that depletion of PKD enhances sensitivity to AR antagonists. The Milestones are nearly achieved. By overexpressing PKD, we showed that the increased PKD expression caused chemoresistance. Data describing these findings will be combined with the *in vivo* data for publication. The manuscript is currently under preparation.  **Figure 4. Overexpression of PKD2 reduced the sensitivity of LNCaP prostate cancer cells to enzalutamide and docetaxel.** Cell transfected with empty vector (EV) and Flag-PKD2 were treated with increasing concentrations of docetaxel and enzalutamide. Cell survival were measured by MTT assay. Data are from 3 experiments .	90% completed
Major Task 4: Test the hypothesis that PKD promotes resistance to antiandrogens through modulating Aurora-A and Cenp-E expression and mitotic programing. **We have made major progress on these studies. The results are described below.**	
Subtask 1: Determine the role of PKD in mitosis. Our study has demonstrated an important role of PKD in G2/M transition and mitotic entry. Our data indicated that PKD, particularly PKD2, was activated during G2 and M phase of cell cycle, and its activity is required for mitotic entry and progression. Inhibition of PKD resulted in mitotic catastrophe. A manuscript that describes these findings has now been published (Roy et al., *Mol Cancer Res*, 2018, 16:1785-1797).	completed
Subtask 2: Determine if Aurora-A and Cenp-E account for the effects of PKD on mitosis. We found that overexpression of Aurora A reversed the effect of PKD inhibitor on cell cycle, thus Aurora A mediated the effects of PKD on G2/M transition and mitotic progression. The findings are described in our publication (Roy et al., *Mol Cancer Res*, 2018, 16:1785-1797).	completed
Subtask 3: Examine a direct role of PKD in regulating Aurora-A and Cenp-E during mitosis through direct binding or phosphorylation. We found that PKD co-localized with Aurora A, but did not directly interact with Aurora A. We also did not detect changes of Aurora A phosphorylation when inhibiting or depleting PKD. The findings are described in our paper (Roy et al., *Mol Cancer Res*, 2018, 16:1785-1797). Due to the large size of Cenp-E which was difficult to study, we sought to focus solely on Aurora A, a key mitotic regulator.	completed

Milestone(s) Achieved: Demonstrate a causal role of Aurora-A and Cenp-E in PKD-regulated mitosis. Our study has demonstrated a causal role of Aurora-A in PKD-regulated mitotic entry. We did not conduct further study on Cenp-E because of technical difficulty (poor antibody and the large size of the protein). We decide to focus our efforts on Aurora A. We also found that PKD has a dominant role in mitotic entry. Our findings are described in our paper (Roy et al., *Mol Cancer Res*, 2018, 16:1785-1797).	completed
Specific Aim 3 Determine the functional input of PKD in ADT resistance *in vivo* and assess the efficacy of PKD SMI in combination with AR antagonists in prostate cancer tumor xenografts	
Major Task 5: Determine that increased PKD1 expression confers resistance to AR antagonists in PrCa xenograft models. **Please see description under each subtask for the progress of the studies.**	
Subtask 1: Establish stable inducible PKD1 knockdown cell lines derived from LNCaP, C4-2, and VCaP cells. We had successfully cloned the PKD1-shRNAs into Doxycycline (Dox)-Inducible RNAi vector. We are still in process of obtaining stable inducible PKD1 knockdown cell lines derived from LNCaP, C4-2, and VCaP cells. Due to difficulty in maintaining the phenotypes of LNCaP and C4-2, we have not obtained stable clones from these cells.	1-6 on-going
Subtask 2: Establish stable PKD1 overexpressing cell lines derived from LAPC4. We have obtained the constructs for overexpression of PKD1 in mammalian cells. We are in the process of establishing the PKD1 overexpressing cell lines in LAPC4. We encountered similar difficulties as described above in Subtask 1.	1-3 on-going
Subtask 3: Conduct s.c. tumor xenograft studies on the stable cell lines established above. This subtask has not been completed. The work will be initiated once we have stable cell lines established.	1-6 On-going
Milestone(s) Achieved: Obtain the stable cell lines with knockdown or overexpression of PKD. Establishing stable PKD knockdown or overexpressing cell lines in androgen-sensitive PrCa cells (LNCaP and LAPC4) has been difficult, because of the unstable phenotypes of these cell lines. We have adopted a different strategy by establishing PKD1-KO cells by CRISPR/Cas9. This is currently on-going.	1-6 on-going
Major Task 6: Determine that targeted inhibition of PKD by CRT101 enhances the efficacy of AR antagonists *in vitro* and *in vivo* **Please see description under each subtask for the progress of the studies.**	
Subtask 1: Determine the efficacy of CRT101 and enzalutamide alone and in combination on the growth of VCaP prostate tumor xenografts.	1-12

We have completed the testing VCaP and 22Rv1 cells for sensitivity to CRT101 +/- enzalutamide. The tumor xenograft study is still on-going. In addition to the tumor xenograft study, we will also test the combination in TRAMP mice (an immunocompetent autochthonous prostate cancer model). We are currently breeding the TRAMP for *in vivo* testing of the drug combination. This work will be completed in the next 6-12 months.	on-going
Subtask 2: Analysis of tumor tissues and biomarkers. This subtask has not been completed. The study will be done along with Subtask1 in the new funding cycle.	1-6 on-going
Milestone(s) Achieved: Demonstrate synergy between CRT1010 and enzalutamide in PrCa xenograft models and TRAMP mice. We have demonstrated the synergistic effects of CRT1010 and enzalutamide in PrCa cell lines. We are in the process of evaluating the combination in xenograft models and TRAMP prostate cancer mouse model.	6-12 on-going
OTHER ACHIECEMENTS We are investigating the role of PKD1 in prostate cancer metastasis, a key feature of mCRPC that directly contributes to the high mortality of this malignant disease. We have successfully established a bone metastasis mouse model by intracardiac injection of metastatic PC3-ML cells. We have completed the *in vivo* efficacy analysis of a PKD inhibitor - CRT101 on prostate cancer metastasis to the bone. The mechanistic studies in cell lines are also near completion. A manuscript that describes these findings is currently under preparation.	

What opportunities for training and professional development has the project provided?

The project provided opportunities for training postdoctoral fellows.
Since October 2011, all postdoctoral trainees in the schools of the health sciences at the University of Pittsburgh have been required to complete an annual career development plan (also known as an individual development plan - IDP) as part of our institution's Postdoctoral Career Development and Progress Assessment Process.
This process, overseen by the Center for Postdoctoral Affairs in the Health Sciences, requires that a postdoc work with his or her faculty mentor to establish an annual career development plan and to also identify two additional individuals to serve as members of the postdoc's mentoring team. The postdoc also completes an annual self-assessment relative to his or her career development plan which contributes in part to the faculty mentor's annual assessment of the postdoc's progress towards his or her career goals.

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

4. During six-month extension of this project, our main goal is to complete the in vivo study proposed in Specific Aim 3 and submit our new manuscript. Although we have demonstrated that overexpression of PKD conferred resistance to AR antagonists, it is not known if it also occurs *in vivo*. We are in the process of evaluating the efficacy of CRT101 and its combination with AR antagonists in s.c. tumor xenograft model and the TRAMP mouse model. If time allows, we will also examine the combination in the mouse PC3-ML bone metastasis model we established previously. Bone is a major metastatic site of prostate cancer. The inhibition of bone metastasis by CRT101 will have significant clinical implication for prostate cancer therapy.

What was the impact on the development of the principal discipline(s) of the project?

This application fills an important knowledge gap in signaling mechanisms and therapeutic targeting of PKD in the context of ADT-induced therapy resistance in prostate cancer. (**1**) The successful completion of the study will provide mechanistic insights to AR-regulated prosurvival pathways centered on PKD in therapy resistance and tumor progression. (**2**) PKD may be targeted to enhance the therapeutic efficacy of antiandrogens and other chemotherapeutic agents, indicating the significant translational value of the study. (**3**) The identification of AURKA as biomarkers of PKD SMIs may facilitate the translation of these agents to the clinic. (**4**) AURKA may be used for identifying PKD SMI-sensitive prostate tumor subtypes and facilitate the application of PKD-targeted agents in personalized therapy.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications.

  1. Roy, A., Prasad, S., Zhao, J., and *Wang, Q. J. Protein kinase D promotes prostate cancer cell bone metastasis by positively regulating Runx2 in a MAPK-ERK1/2-dependent manner. Manuscript under preparation.
  2. Tandon M., Nagpal I., Roy, A., and *Wang, Q. J. Inhibition of PKD synergizes with androgen receptor antagonists in suppression of prostate cancer growth and metastasis. Manuscript under preparation.

  Books or other non-periodical, one-time publications.

  Nothing to Report.

Other publications, conference papers, and presentations.

"Protein Kinase D 2 Promotes Cell Cycle Progression by Stabilizing Aurora A Kinase at Centrosome in Cancer Cells", FASEB SRC: The Protein Kinases and Protein Phosphorylation Conference, poster presentation, 2019 July.

- **Website(s) or other Internet site(s)**

Nothing to Report.

- **Technologies or techniques**

Nothing to Report.

- **Inventions, patent applications, and/or licenses**

Nothing to Report.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Qiming Wang
Project Role: Principal Investigator
Research Identifier (e.g. ORCID ID):
Nearest Person Month Worked: 2
Contribution to the project: the overall scientific management of this application, including experimental design, data collection and analysis, supervision of postdoctoral research associates, preparation and submission of reports and manuscripts.
Funding Support: DOD PC150190

Name: Manuj Tandon
Project Role: Research Instructor
Research Identifier (e.g. ORCID ID):
Nearest Person Month Worked: 6
Contribution to the project: overall execution of the project, data collection, and analysis; writing of reports and papers.
Funding Support: DOD PC150190

Name: Sahdeo Prasad, Adhiraj Roy
Project Role: Postdoctoral Associate
Research Identifier (e.g. ORCID ID):
Nearest Person Month Worked: 8
Contribution to the project: technical assistance in execution of experiments, data analysis, and human/mouse tissue pathohistological analysis.
Funding Support: DOD PC150190

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Dr. Wang has been awarded a R01 (R01CA229431) and an AHA (19TPA34850096) grant starting 7/1/2019.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Nothing to Report.

QUAD CHARTS:

Nothing to Report.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Two manuscripts are included in the appendices.

www.impactjournals.com/oncotarget/ Oncotarget, 2017, Vol. 8, (No. 8), pp: 12800-12811

Research Paper

Androgen suppresses protein kinase D1 expression through fibroblast growth factor receptor substrate 2 in prostate cancer cells

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Keywords: *androgen, androgen-repressed genes, androgen receptor, protein kinase D1, prostate cancer*

Received: *August 21, 2016* ***Accepted:*** *December 27, 2016* ***Published:*** *January 06, 2017*

ABSTRACT

In prostate cancer, androgen/androgen receptor (AR) and their downstream targets play key roles in all stages of disease progression. The protein kinase D (PKD) family, particularly PKD1, has been implicated in prostate cancer biology. Here, we examined the cross-regulation of PKD1 by androgen signaling in prostate cancer cells. Our data showed that the transcription of PKD1 was repressed by androgen in androgen-sensitive prostate cancer cells. Steroid depletion caused up regulation of PKD1 transcript and protein, an effect that was reversed by the AR agonist R1881 in a time- and concentration-dependent manner, thus identifying PKD1 as a novel androgen-repressed gene. Kinetic analysis indicated that the repression of PKD1 by androgen required the induction of a repressor protein. Furthermore, inhibition or knockdown of AR reversed AR agonist-induced PKD1 repression, indicating that AR was required for the suppression of PKD1 expression by androgen. Downstream of AR, we identified fibroblast growth factor receptor substrate 2 (FRS2) and its downstream MEK/ERK pathway as mediators of androgen-induced PKD1 repression. In summary, PKD1 was identified as a novel androgen-suppressed gene and could be downregulated by androgen through a novel AR/FRS2/MEK/ERK pathway. The upregulation of prosurvival PKD1 by anti-androgens may contribute to therapeutic resistance in prostate cancer treatment.

INTRODUCTION

Prostate cancer is the most common noncutaneous malignancy and the second leading cause of cancer-related deaths among men in the United States. The initiation and progression of prostate cancer is uniquely dependent on androgen receptor (AR)-induced signaling. Although androgen deprivation therapy provides an initial favorable response in advanced prostate cancer, the more aggressive castration-resistant prostate cancer (CRPC) develops invariably in almost all patients, eventually leading to death. It has become increasingly clear that continuous activation of the AR in CRPC remains the main driving force of tumor progression and metastasis. Thus, understanding the critical events associated with the AR signaling is essential for developing novel and effective therapies to treat CRPC.

The protein kinase D (PKD) family of serine/threonine kinases belongs to the Ca^{2+}/calmodulin-dependent protein kinase (CAMK) superfamily [1, 2]. To date, three isoforms of PKD have been identified, PKD1 (formerly PKCμ) [3, 4], PKD2 [5], and PKD3 (formerly PKCν) [6]. In intact cells, PKD activation involves phosphorylation of two conserved serine residues in the activation loop by DAG-responsive PKCs [7–9], and PKD activity can be maintained independently of PKC through autophosphorylation [10, 11]. Emerging evidence supports that PKD has an important role in carcinogenesis

and tumor progression [12, 13]. A recent report suggested that a hotspot activating mutation in *PRKD1*, the gene encoding PKD1, may drive polymorphous low-grade adenocarcinoma (PLGA), the second most frequent type of malignant tumor of the minor salivary glands [14]. PKD regulates a variety of tumor-associated biological processes, including tumor cell proliferation, growth, survival, migration, invasion, secretion, and angiogenesis [12, 15–20]. Aberrant PKD activity and expression have been demonstrated in tumor cell lines and tumor tissues from the pancreas [18], skin [19, 21], breast [22], and prostate [20, 23]. In particular, PKD has been shown to play an important role in the pathogenesis of prostate cancer [20, 24–26], and targeted PKD inhibition potently blocks prostate cancer cell proliferation and survival [26, 27].

Fibroblast growth factor (FGF) signaling is a highly complex signaling network that comprises 18 ligands, which bind to and activate four highly conserved transmembrane tyrosine kinase receptors (FGFR1, FGFR2, FGFR3, and FGFR4).The FGF/FGFR pathway plays an important role in cancer development and progression by modulating a variety of biological processes, including cell proliferation, survival, and migration [28, 29]. FGFR substrate 2 (FRS2/FRS2α), also known as FGFR-signaling adaptor SNT1 (suc1-associated neurotrophic factor target 1), is regarded as the 'conning center' for intracellular signaling elicited by the activation of FGFRs at the cell surface. FRS2 forms complexes with Grb2-Sos and Grb2-Gab1 to activate the Ras/Raf/MEK/ERK and PI3K/Akt pathways [29, 30]. Although FRS2 expression is not regulated by androgen [31], androgen-sensitive prostate cancer cells express FGF2, and its expression is upregulated in response to androgen stimulation [32]. Thus, androgen regulates the activity of FGFR signaling in prostate cancer cells.

In this study, we report for the first time that PKD1 was tightly regulated by androgen at the transcriptional level in prostate cancer cells and was a novel androgen-repressed gene. Inhibition or knockdown of androgen receptor (AR) blocked androgen depletion-induced PKD1 expression, indicating that AR was required for the repression of *PRKD1* gene expression. Further analysis identified FRS2 as a novel mediator of androgen-induced PKD1 repression. The regulation of PKD1 by androgen and AR may have important implications in the therapeutic response to AR-targeted agents.

RESULTS

Androgen repressed PKD1 expression in androgen-sensitive prostate cancer cells

Androgen signaling plays a crucial role in prostate cancer initiation and progression. In this study, we sought to determine whether androgen modulated PKD1 expression and signaling. PKD1 was detected in androgen-sensitive LNCaP cells and two castration-resistant LNCaP-derivative cell lines, C4-2 (androgen-hypersensitive) and C81 (androgen-insensitive), but not in androgen-sensitive LAPC4 cells. As shown in Figure 1A, a significant increase in PKD1 expression was observed upon androgen depletion (AD) in LNCaP and C4-2 cells and to a lesser extent in C81 cells. R1881, a synthetic androgen agonist, induced remarkable concentration-dependent suppression of PKD1 expression at the transcript (Figure 1B) and protein (Figure 1C) levels in LNCaP and C4-2 cells. R1881 also suppressed PKD1 expression in VCaP cells, a castration-resistant prostate cancer cell line that expresses wild-type AR, in a concentration-dependent manner (Figure 1D). Interestingly, PKD2 expression was similarly suppressed by R1881 in a concentration-dependent manner in LNCaP and VCaP cells (Supplementary Figure 1A–1B). PKD3 was also upregulated upon androgen withdraw in LNCaP cells, despite its low endogenous expression (Supplementary Figure 1A). In contrast, androgen did not affect the expression of PKD1 and PKD2 in another castration-resistant cell line, 22Rv1, which expresses both full-length AR and truncated AR variants (Supplementary Figure 1C), suggesting that the effect of androgen may be cell context-dependent. Taken together, we concluded that PKD1 was an androgen-repressed gene.

PKD1 expression was dependent on the induction of a repressor protein

The kinetics of PKD1 regulation in response to androgen deprivation or R1881 treatment was examined. As shown in Figure 2A, androgen deprivation gradually up regulated PKD1 protein expression, which peaked at 16–24 h, while R1881 suppressed PKD1 expression with similar kinetics. The induction of PKD1 transcript and its inhibition by R1881 correlated well with the time-course of protein expression (Figure 2B).

To gain insights into the regulation of PKD1 by androgen, we first examined whether R1881 affected PKD1 mRNA stability. The half-life (t½) of PKD1 mRNA was determined in the presence of actinomycin D, an inhibitor of gene transcription. As shown in Figure 2C, the t½ of PKD1 mRNA was about 4 h, which was not significantly altered by the addition of R1881 ($p > 0.5$), indicating that R1881 did not impact the stability of PKD1 mRNA. Next, cycloheximide (CHX) was used to inhibit protein synthesis to determine whether the regulation of PKD1 gene expression by androgen involved *de novo* protein synthesis. CHX induced a nearly 2-fold increase in PKD1 expression and completely blocked R1881-induced PKD1 downregulation, indicating that the suppression of PKD1 expression likely required the induction of a repressor protein (Figure 2D). This finding was in line with the gradual onset of PKD1 regulation by androgen, further supporting the involvement of a repressor protein. Taken together, our data indicated that androgen-regulated PKD1 expression was dependent on the presence of a repressor protein.

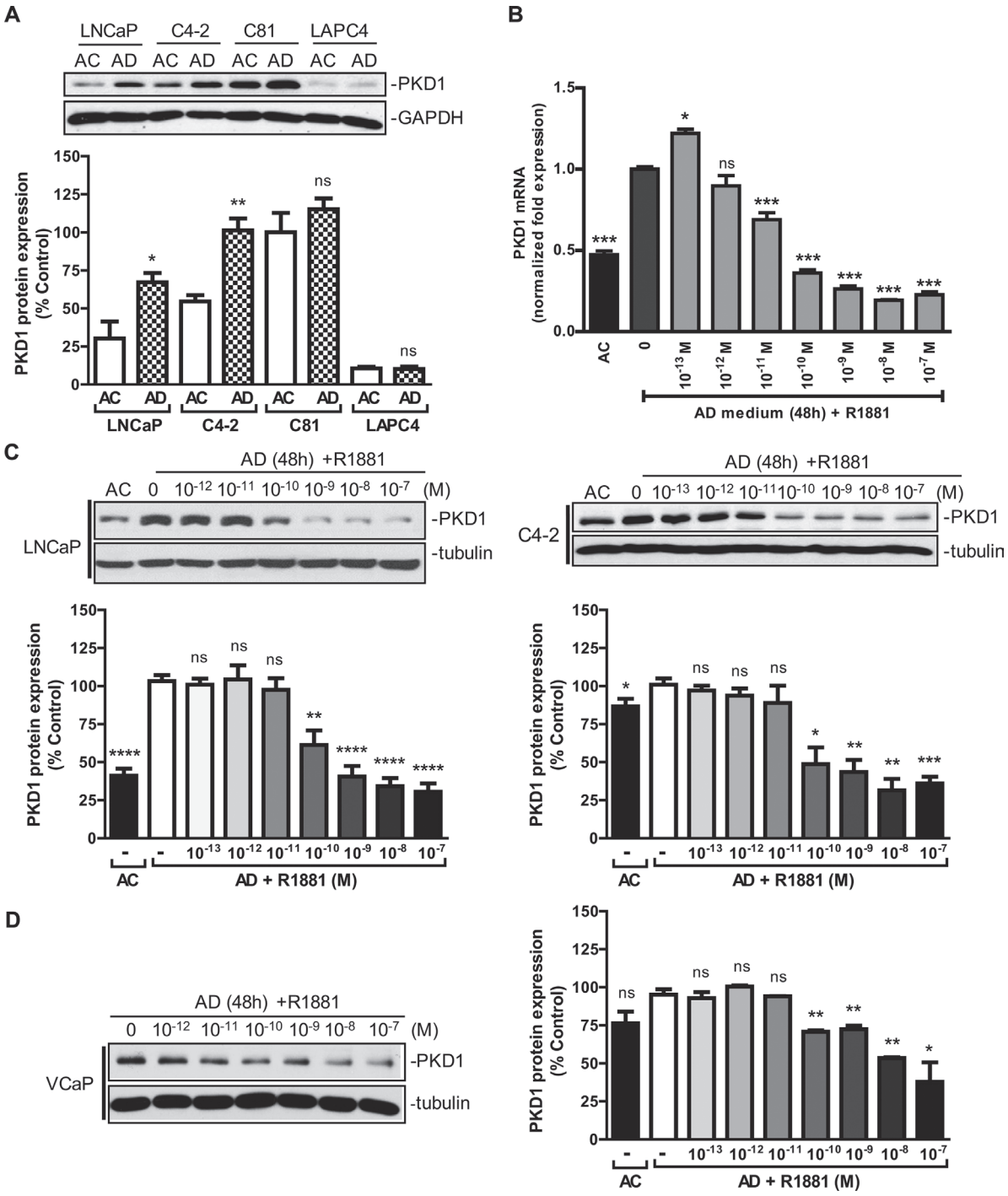


Figure 1: Androgen repressed PKD1 expression. (**A**) Effects of androgen depletion on PKD1 expression in prostate cancer cells. LNCaP, C4-2, C81, and LAPC4 cells were grown for 48 h in normal androgen-containing (AC) or androgen-depleted (AD) medium supplemented with charcoal-stripped FBS. Cells were lysed and subjected to immunoblotting for PKD1 and GAPDH (loading control). *Bottom*, quantitative measurement of band intensity by densitometry analysis. The data were expressed as % control with C81 (AC) set as 100%. Data are the mean ± SEM of four independent experiments. (**B**) Androgen inhibited PKD1 transcription. Total RNAs from LNCaP were extracted, and real-time RT-qPCR was conducted using specific PKD1 primers. *GAPDH* was used as internal control. Data are the mean ± SEM of three independent experiments. (**C**) Androgen suppressed PKD1 protein expression. LNCaP and C4-2 cells were grown in androgen-depleted medium for 48 h, following by treatment without or with increasing concentrations of androgen R1881. Cells were harvested after 24 h and subjected to immunoblotting. *Bottom*, the band intensity was quantified by densitometry analysis, and data are the mean ± SEM of ten (LNCaP) or three (C4-2) independent experiments. (**D**) Androgen suppressed PKD1 protein expression in castration-resistant VCaP cells. VCaP cells were grown in androgen-depleted medium for 48 h, followed by treatment without or with androgen R1881 for 24 h. Cells were harvested for immunoblotting. Data from one of three independent experiments are shown. *Right*, quantitative measurement of band intensity from three experiments is shown. ns, not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

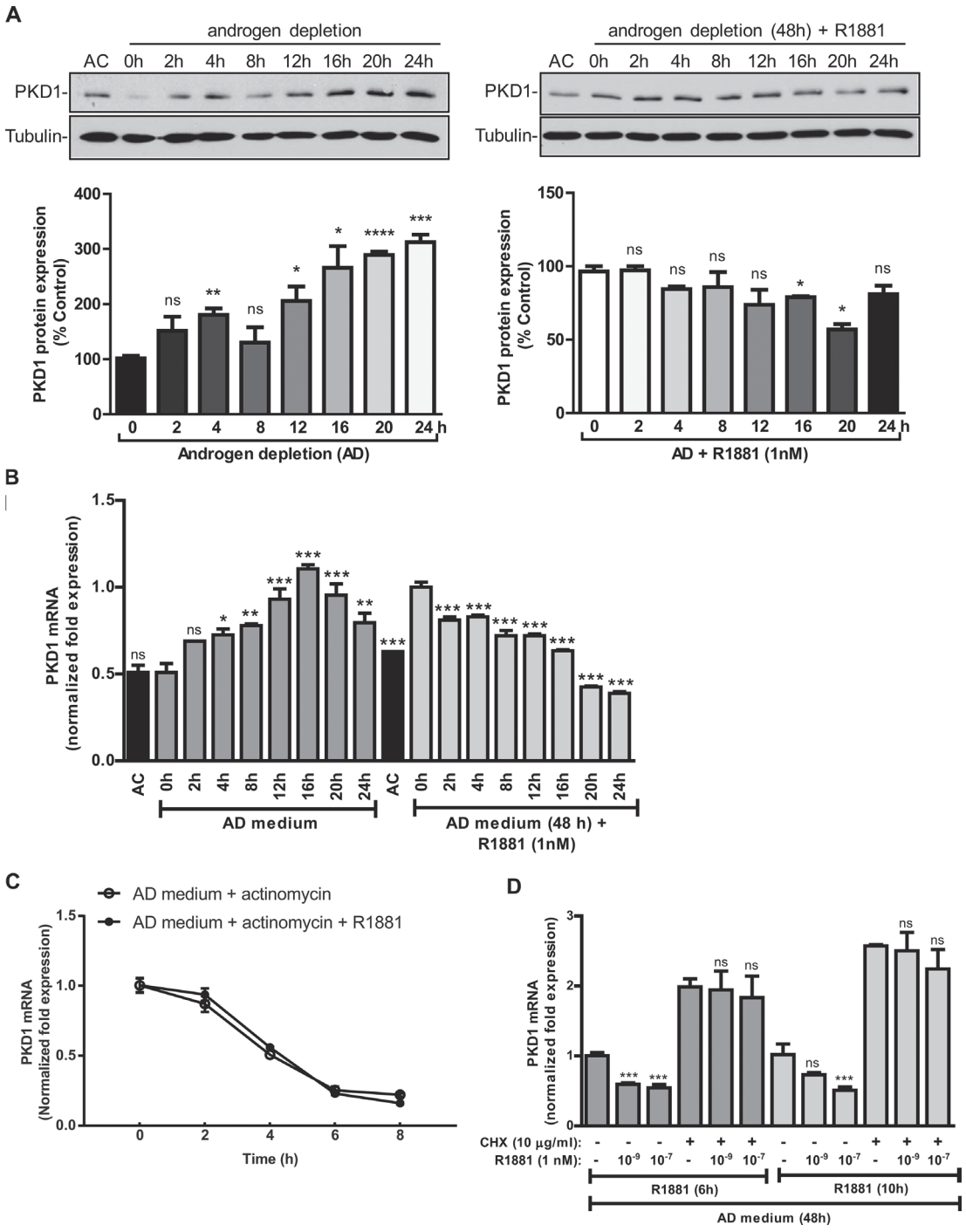


Figure 2: PKD1 expression was dependent of the induction of a repressor protein. (**A**) Kinetics of PKD1 regulation by androgen. *Left panels*, LNCaP cells were grown in AD medium for the indicated times. *Right panels*, LNCaP cells were grown in AD medium for 48 h, followed by treatment with R1881 (1 nM) for the indicated times. Cells were harvested and subjected to immunoblotting for PKD1 and tubulin (loading control). Cells grown in AC medium were used as the control. Representative data from one of four experiments are shown. *Bottom*, quantitative measurement of band intensity by densitometry analysis. Data are the mean ± SEM of three independent experiments. (**B**) Kinetics of PKD1 transcript expression. LNCaP cells were treated as above in "A". Total RNAs were extracted, and the kinetics of PKD1 mRNA induction/suppression were examined by real time RT-qPCR. (**C**) R1881 did not affect PKD1 mRNA stability. LNCaP cells were grown either in AD medium for 48 h, followed by the addition of actinomycin D (2 ng/mL) with or with R1881 (1 nM) for the indicated times. Total RNAs were extracted and subjected to real time RT-qPCR for analysis of PKD1 transcripts. Not significant by paired t test ($p > 0.5$). (**D**) PKD1 expression required the induction of a repressor protein. LNCaP cells were grown in AD medium for 48 h, followed by R1881 treatment with or without cycloheximide (CHX) for 6 or 10 h. Total RNAs were extracted, and the levels of PKD1 mRNA were measured by real-time RT-qPCR. *GAPDH* was used as a loading control. Data are the mean ± SEM of at least three independent experiments. ns, not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

AR mediated PKD1 repression by androgen

Androgens are important hormones for normal physiology and are responsible for certain disease conditions. Their actions are mediated by the AR, a ligand-dependent nuclear transcription factor. Androgens binds to AR after entering the cells to form an androgen-receptor complex, which then translocates to the nucleus where it binds to androgen response elements (AREs) in the promoter regions and regulates the transcription of its target genes. The actions of AR can be blocked by AR inhibitors, such as bicalutamide (Casodex) or enzalutamide (MDV3100). Bicalutamide is known to bind AR and leads to the formation of a transcriptionally inactive AR complex [33]. In this study, we sought to examine whether AR was required for the repression of PKD1 expression by R1881, and bicalutamide was used to determine whether the inhibition of AR activity affected PKD1 expression. After androgen deprivation, LNCaP and C4-2 cells were treated with R1881 at 1 nM in the presence or absence of bicalutamide (10 μM). As shown in Figure 3A, bicalutamide significantly reversed R1881-induced PKD1 repression in LNCaP and C4-2 cells. In LNCaP cells, inhibition of AR by bicalutamide also upregulated PKD1 protein expression in a concentration-dependent manner (Figure 3B). The specific role of AR was then examined using multiple AR-targeted siRNAs. Our data showed that knockdown of AR by three siRNAs targeting different regions of the AR transcript significantly blocked R1881-induced PKD1 suppression in LNCaP (Figure 3C) and C4-2 cells (data not shown). AR knockdown was confirmed by western blotting. Taken together, these data suggested that AR was required for the transcriptional repression of PKD1 gene expression caused by androgen stimulation.

To determine whether AR directly regulated the expression of PKD1, we analyzed the promoter region of PKD1, which led to the identification of two potential AREs upstream of the transcription start site (TSS). The human PKD1 gene spans ~45.7 kb. Analysis of up to 5000 bp of the promoter region upstream from the TSS revealed two putative AREs. (ARE1, 5′-AGTACTTTAAGCTCT-3′; ARE2, 5′-AGAACAAAATAAGCT-3′; (Supplementary Figure 2A). The regions (pm1 and pm2) that contained the AREs were separately cloned into the pTA-Luc reporter. Their activities were analyzed in LNCaP cells cultured in the presence or absence of androgen depletion, followed by treatment with or without R1881. Our data indicated that no luciferase activity was detected from both reporters in LNCaP cells (Supplementary Figure 2B), implying that the AREs in PKD1 promoter did not play an active role in regulating PKD1 transcription in response to androgen stimulation.

An AR co repressor screen revealed FRS2 as the potential mediator of androgen-induced PKD1 repression

The involvement of AR and an androgen-induced repressor protein prompted us to conduct an esiRNA screen that targeted 23 AR corepressors and other related proteins. LNCaP cells were transfected individually with 23 esiRNAs, followed by androgen depletion and treatment with or without R1881. Levels of PKD1 transcript were analyzed by real time RT-qPCR. In the controls, androgen depletion induced PKD1 expression, and treatment with R1881 caused over 2-fold reduction in PKD1 mRNA. As shown in Figure 4, similar to the non targeting siRNA, R1881-induced PKD1 repression was not affected by the depletion of all target genes, with the exception of FRS2. Knockdown of FRS2 by esiRNA completely reversed the repression of PKD1 transcription by R1881 (Figures 4C, 5A). In summary, FRS2 was identified as a potential repressor of PKD1 gene expression.

Androgen repressed PKD1 expression through a FGFR/FRS2/MEK/ERK pathway

The role of FRS2 was further validated using FRS2 siRNAs (si-FRS2-1, -2). Depletion of FRS2 abolished the R1881-induced suppression of PKD1 transcription, confirming FRS2 as a potential mediator of androgen-dependent PKD1 repression (Figure 5A). Furthermore, at the protein level, knockdown of FRS2 by two different siRNAs completely abrogated the downregulation of PKD1 by R1881 (Figure 5B). *FRS2* siRNAs caused significant knockdown of *FRS2* mRNA (Figure 5C). Thus, FRS2 mediated androgen-induced PKD1 repression.

The adaptor protein FRS2 is a major mediator of the FGFR signaling in normal and malignant cells. FGFR stimulation by FGF leads to the tyrosine phosphorylation of FRS2, which then forms a complex with Grb2 and Sos to activate the downstream Ras/Raf/MEK/ERK signaling pathway. Androgen-sensitive LNCaP cells express low levels of FGF2, and its expression is upregulated in response to androgen stimulation [32]. Here, we sought to determine whether the FRS2-mediated FGFR signaling pathway was involved in the regulation of PKD1 by androgen. As shown in Figure 5D, PD173074, an inhibitor of FGFR, significantly reversed R1881-induced PKD1 repression, indicating that FGFR activity was required for the inhibition of PKD1 by androgen/AR. Next, the role of the FGF-activated MEK/ERK MAPK signaling pathway was evaluated. Our data demonstrated that R1881-induced PKD1 suppression was abrogated in a concentration-dependent manner by UO126, a MEK inhibitor. Thus, MEK/ERK activity was also required for the suppression of PKD1 by androgen (Figure 5E). Since the suppression of PKD1 is likely associated with the secretion of a

FGFR ligand, we tested the effects of inhibiting secretory pathways on the expression of PKD1 using brefeldin A (BFA), a fungal metabolite and an inhibitor of intracellular protein transport that inhibits constitutive secretion from the *trans*-Golgi network. Our data indicated that BFA at 5 and 10 μM completely reversed androgen-induced PKD1 suppression (Figure 5F). In summary, our data implied that androgen suppressed PKD1 expression through an indirect FGFR/FRS2/MEK/ERK pathway in prostate cancer cells.

DISCUSSION

In this study, we present the novel findings demonstrating that PKD1 was repressed by androgen/AR at the mRNA and protein levels in androgen-sensitive prostate cancer cells, identifying PKD1 as a novel androgen-repressed gene. We further identified FRS2 as a novel mediator of androgen-induced repression of PKD1 expression. The cross-regulation of PKD1 by androgen/AR places PKD1 in the AR-induced signaling

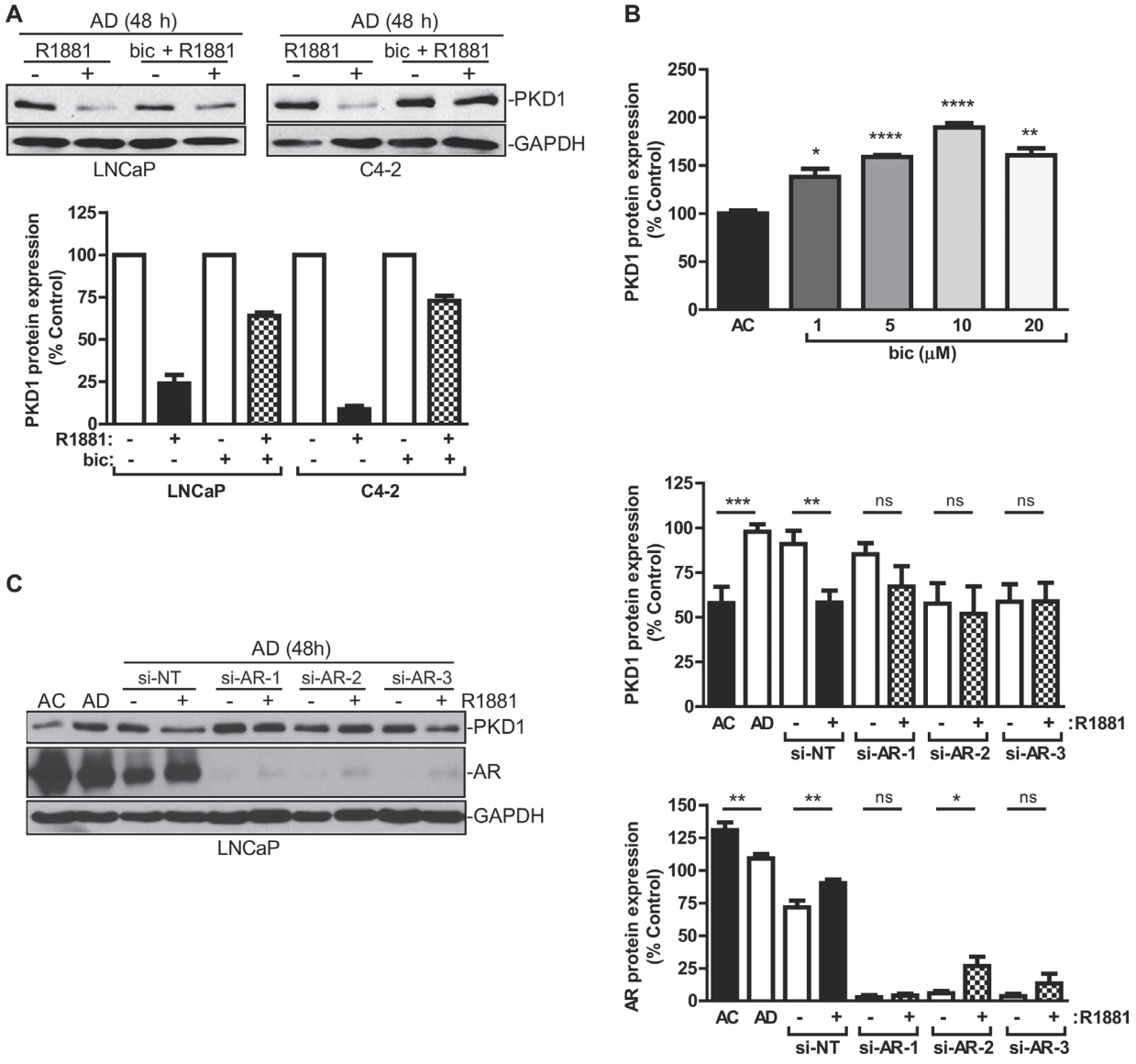


Figure 3: AR mediated PKD1 repression by androgen. (**A**) AR inhibition led to increased PKD1 expression. LNCaP and C4-2 cells cultured in AD medium for 48 h were treated with or without R1881 (1 nM) ± bicalutamide (Bic) (10 μM) for 16 h. *Bottom*, quantitative measurement of band intensity from two experiments. (**B**) Bicalutamide caused PKD1 upregulation. LNCaP cells were treated with increasing concentrations of bicalutamide for 48 h, followed by immunoblotting for PKD1. The band intensity was quantified by densitometry analysis, and data are the mean ± SEM of three independent experiments. (**C**) AR was required for transcriptional regulation of PKD1 by androgen. LNCaP cells were transfected with non targeting siRNA (si-NT) or AR siRNAs (si-AR-1, -2, -3). After 48 h, the medium was replenished with AD medium with or without R1881 (1 nM) for 16 h. Cells were collected and subjected to immunoblotting for PKD1, AR, and GAPDH. *Right*, quantitative measurement of band intensity for PKD1 (*top*) and AR (*bottom*) from three experiments is shown. ns, not significant; **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. Data are the mean ± SEM of six independent experiments.

network, which is critical to prostate cancer progression. The transcriptional regulation of PKD isoforms has not been studied in the past. Our study provides the first mechanistic understanding of a novel androgen-induced AR/FRS2/MEK/ERK pathway that regulates the expression of PKD1. As a well-documented prosurvival signaling protein, PKD1 upregulation in response to androgen deprivation and anti-androgen treatment may have significant implications in therapy resistance and progression to CRPC.

The class I steroidal nuclear receptor AR is a critical regulator of tumor initiation and progression in both early and advanced prostate cancer. As a transcription factor, AR exerts its actions mainly through regulating the expression of a host of target genes. Among them, AR-stimulated genes have been extensively studied, with prostate-specific antigen (PSA) being the best characterized. In contrast, AR-repressed target genes have not been well characterized. These genes constitute a large portion of AR-targeted genes, and some have been shown to play essential roles in prostate cancer progression [34, 35]. Diverse mechanisms have been proposed to account for the repression of target gene expression by AR. These include both genomic mechanisms, such as active repression via the recruitment of corepressor complexes, and nongenomic mechanisms, such as regulation of signaling pathways [34]. Our data showed that inhibition or silencing of AR blocked the suppression of PKD1 by R1881, indicating that AR was required for the downregulation of PKD1. Initially, analysis of the 5′ promoter region of the PKD1 gene led to the identification of two potential AREs, which prompted us to investigate the direct role of AR in transcriptional repression of the PKD1 gene. However, analysis of the transcriptional activity of the ARE-containing PKD1 promoter failed to detect any androgen-induced transcriptional activities associated with pm1 and pm2, suggesting that the identified potential AREs may be inactive. Although less common, inactive AREs have been demonstrated, even in the presence of AR binding, and more complex mechanisms have been suggested to be involved in the regulation of genes nearby these AREs in prostate cancer cells [36, 37]. Importantly, kinetic analysis demonstrated a slow and gradual onset of PKD1 downregulation at the protein and transcript levels, which peaked at about 16–20 h in response to androgen; this finding also provides evidence against a mechanism involving active transcriptional repression through direct interaction with AREs. The involvement of an androgen-induced repressor

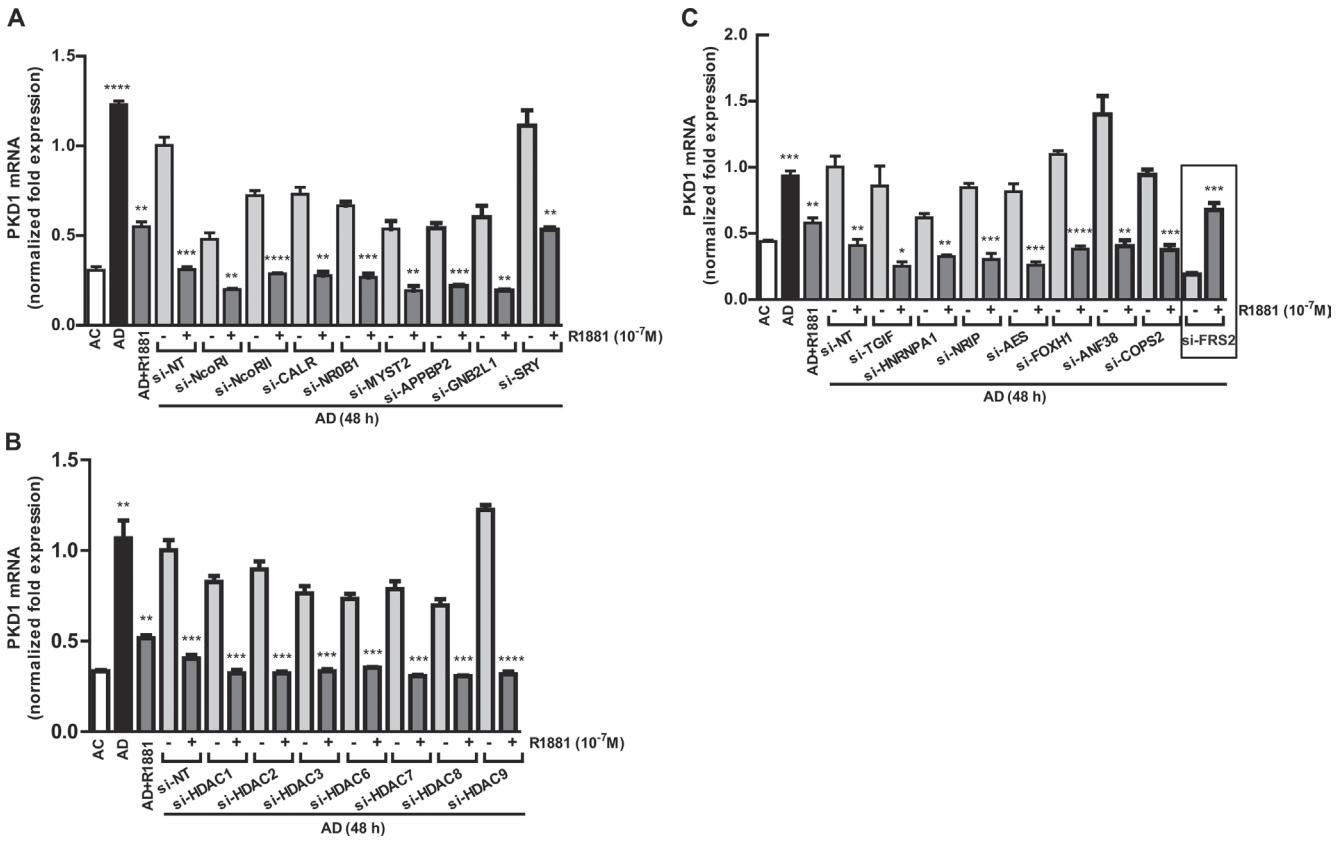
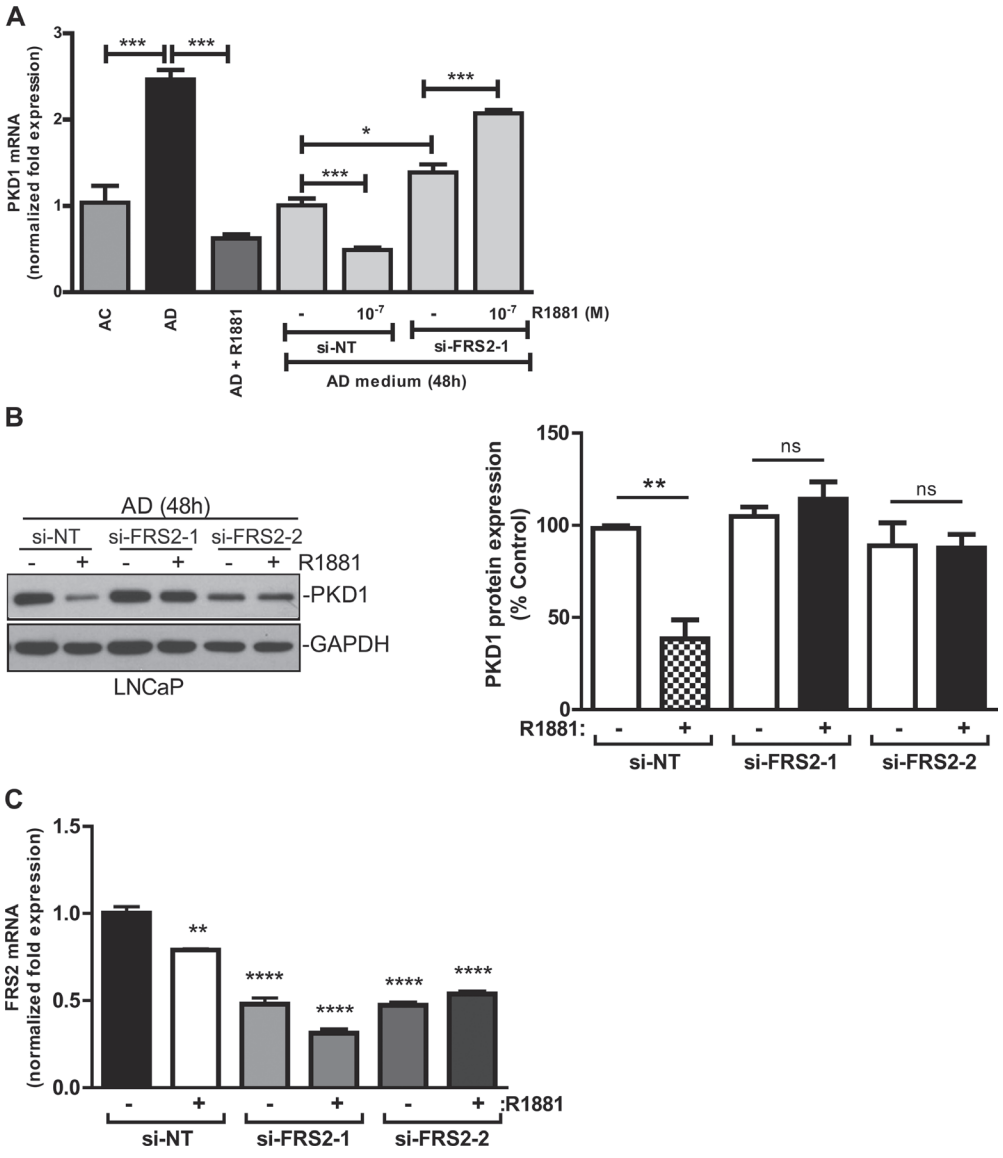


Figure 4: Screening of AR corepressors. (**A**–**C**) An esiRNA screen that targeted 23 AR corepressors and other related proteins was conducted in LNCaP cells. The cells transfected with esiRNAs were subjected to androgen depletion for 48 h, followed by treatment with or without R1881. Levels of PKD1 transcript was analyzed by real time RT-qPCR. Non targeting siRNA (si-NT) was used as the control. Student's *t*-tests were used to determine the statistical significance between the untreated and R1881-treated groups within each pair of esRNA knockdown samples. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

is supported by the data showing that CHX abolished androgen-induced PKD1 repression. Although this may also occur without the synthesis of a repressor, for example, the suppressive effects of AR could be mediated through its interaction with a pre-existing labile protein at the AR-repressed loci; CHX treatment will similarly abolish the repressive effect mediated by this labile protein. Certainly, our findings do not exclude the possibility that there may be distal ARE sites that bind to AR and contribute to AR-mediated PKD1 repression. Overall, our current data support an AR-mediated indirect mechanism involving the cell surface adaptor protein FRS2 in the repression of PKD1 by androgen. These findings were based on an unbiased RNAi screen of a library of AR corepressor proteins. Further analysis validated the role of FRS2, as well as its upstream FGFR and the downstream MEK/ERK pathway, in the regulation of PKD1 by androgen.

In androgen-sensitive prostate cancer cells, depletion of FRS2 blocked R1881-induced PKD1 suppression at both the transcriptional and protein levels. Additionally, inhibition of FGFR and MEK, as well as protein secretion, blocked R1881-induced repression of PKD1. Thus, androgen may repress PKD1 through an AR-induced FGFR/FRS2/MEK/ERK pathway to inhibit PKD1 expression in prostate cancer cells. A previous study showed that FRS2 expression is not regulated by androgen in LNCaP cells [31]. However, in androgen-sensitive LNCaP cells, low levels of FGF2 are detected, and the expression of FGF2 is upregulated in response to androgen stimulation [32]. Additionally, androgen stimulates the activity and production of FGF2 and FGF-binding protein in PC3 prostate cancer cells with stably overexpressed AR [38]. In a different study, however, Kassen et al. showed that FGF2 is not expressed, and androgen in turn acts by increasing the bioavailability of FGF2 by releasing trapped FGF2 from the extracellular matrix through activation of heparinase, which leads to activation of FGFR and stimulation of LNCaP cell proliferation



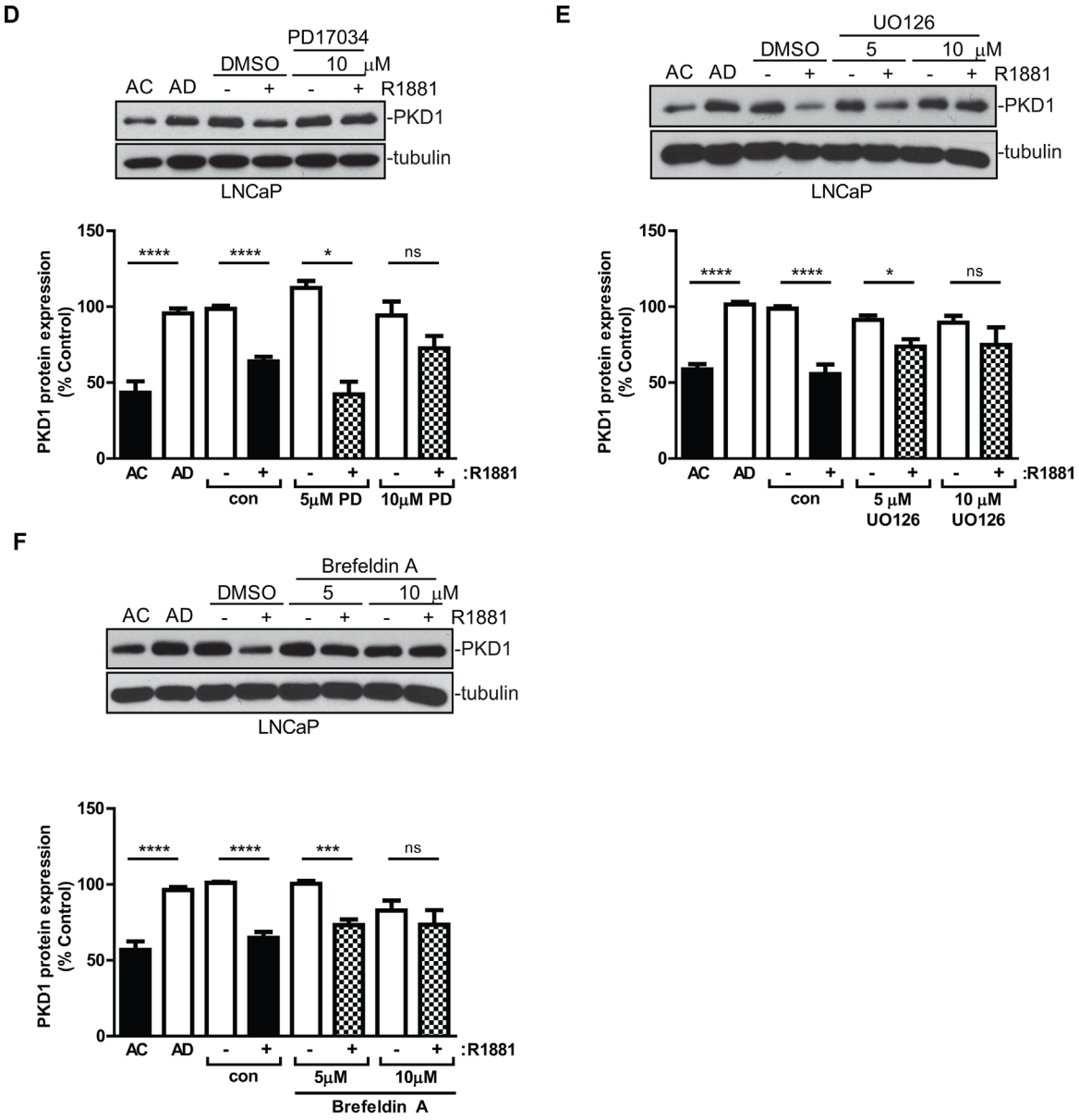


Figure 5: FRS2 was required for androgen-induced PKD1 repression. (**A**) Knockdown of FRS2 reversed androgen-induced repression of PKD1 transcription. Cells were transfected with FRS2 siRNA (si-FRS2-1) and a non targeting siRNA (si-NT), followed by treatment with or without R1881. PKD1 transcripts were analyzed by real-time RT-qPCR. Representative data from one of three independent experiments with triplicate measurements are shown. (**B**) Knockdown of FRS2 blocked the repression of PKD1 protein by androgen. LNCaP cells were transfected with two different FRS2 siRNAs (si-FRS2-1, -2), followed by treatment with R1881. *Right*, quantitative measurement of band intensity for PKD1 from three experiments is shown. (**C**) Real-time RT-PCR confirmed the knockdown of FRS2. Cells from "B" were subjected to RNA extraction, followed by real-time RT-PCR for levels of PKD1 transcript. (**D**–**F**) Androgen repression of PKD1 was dependent on a secretory pathway involving FGFR and MEK. LNCaP cells were grown in AC or AD medium for 48 h, followed by treatment with or without R1881 in the presence or absence of the FGFR inhibitor PD17034 (D), the MEK inhibitor UO0126 (E), and brefeldin A (F) for 16 h. Cell lysates were subjected to immunoblotting for PKD1. Representative images from one of at least three independent experiments are shown. *Bottom*, quantitative measurement of band intensity by densitometry analysis. Data are the mean ± SEM of five to seven independent experiments.

[39]. Regardless of these discrepancies, in all cases, AR promotes the activation of FGFR in prostate cancer cells, which results in phosphorylation of FRS2 and activation of the downstream Ras/Raf/MEK/ERK signaling pathway. By inhibiting MEK activity, we confirmed the requirement for MEK/ERK signaling activity in the suppression of PKD1 by R1881. This evidence supports the notion that PKD1 is repressed by an AR-induced FGFR/FRS2/MEK/ERK pathway in androgen-sensitive prostate cancer cells. The binding of FGF to FGFR leads to the recruitment

of multiple adaptor proteins, including FRS2, Grbs, Sos, and Gab1, and induces the activation of multiple downstream signaling pathways, including MEK/ERK, PI3K/Akt, PLCγ/PKC, and Stat3 pathways. We must state that although our data demonstrated a major role of the MEK/ERK pathway in the regulation of PKD1 expression by androgen, our data did not completely exclude the potential involvement of other pathways, which will be investigated in our future studies.

Our study identified PKD1 as an androgen/AR-repressed gene and uncovered a novel indirect mechanism through which AR regulates PKD1 expression. Although the functional implication of this regulation in prostate cancer progression is still unclear, PKD1 is an important prosurvival signaling protein in normal and cancer cells that functions by regulating multiple signaling pathways, such as stimulating NF-κB, ERK1/2, and Akt and inhibiting JNK and p38 [20, 25, 40]. This notion is further supported by our previous findings that PKD1 protects androgen-sensitive LNCaP prostate cancer cells from phorbol ester-induced apoptosis [25]. Thus, the upregulation of PKD1 as a result of inhibition or loss of AR may promote tumor cell survival and contribute to therapeutic resistance to AR-targeted agents. This further implies that PKD may represent a viable target for mitigating therapy resistance. In castration-resistant C81, 22Rv1, and VCaP cells, we observed different responses to androgen in terms of PKD1 regulation; although androgen did not affect PKD1 expression in 22Rv1 cells, VCaP cells, which express wild-type AR, did respond to androgen stimulation by downregulating PKD1 in a concentration-dependent manner, and minor effects were also observed in C81. This cell context-dependent responsiveness to androgen may be linked to the activity of the AR/FGFR/FRS2 signaling pathway and variations in the expression of its signaling components.

In summary, our study identified PKD1 as a novel androgen/AR-suppressed gene. The suppression of PKD1 was mediated through an indirect mechanism that involved FRS2, a cell surface adaptor protein that connects FGF/FGFR to the downstream MEK/ERK signaling pathway. Our findings suggested that the prosurvival function of PKD1 may have significant implications in prostate cancer progression and therapy resistance. PKD1 may be targeted to enhance the therapeutic response to anti-androgens in prostate cancer treatment.

MATERIALS AND METHODS

Reagents and antibodies

The synthetic androgen methyl trienolone (R1881) was obtained from Perkin-Elmer Life Sciences (Boston, MA), and bicalutamide was purchased from Enzo Life Sciences (Farmingdale, NY). Charcoal-treated fetal bovine serum (FBS) was from Hyclone (Logan, UT) and Sigma (St. Louis, MO). Other cell culture reagents and media were from American Type Culture Collection (ATCC; Rockville, MD). Anti-PKD1, anti-PKD2, and anti-AR antibodies were purchased from Cell Signaling Technology (Danvers, MA). Antibodies targeting GAPDH and α-tubulin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Goat anti-rabbit and goat anti-mouse horseradish peroxidase-conjugated secondary antibodies were from Promega (Madison, WI).

Cell culture and siRNA transfection

LNCaP, C4-2, C81, VCaP, and PC-3 cells were obtained from ATCC (Manassas, VA) and were cultured according to the manufacturer's recommendations. LNCaP cells were discarded after 12 passages. The 23 AR corepressor esiRNAs were obtained from Sigma-Aldrich (Supplementary Table 1). The non targeting siRNA and AR and FRS2 siRNAs were obtained from Integrated DNA Technologies (Coralville, IA). The esiRNAs and siRNAs were transfected into cells using DharmaFECT reagent according to the manufacturer's instructions (GE Dharmacon, Lafayette, CO).

Western blot analysis

Cells were collected and lysed in IP lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 5 mM EGTA, 20 μM leupeptin, 1 mM AEBSF, 1 mM $NaVO_3$, 10 mM NaF, and 1× protease inhibitor cocktail). Lysate protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher, Hudson, NH). Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% milk, the membranes were incubated with primary antibodies in blocking buffer at 4°C overnight. After washing, the membranes were incubated with secondary antibodies at room temperature for 1 h. Protein bands were detected using an enhanced chemiluminescence (ECL) kit. Anti-α-tubulin or anti-GAPDH antibodies were used as a loading control. Densitometry analyses were performed with ImageJ software (NIH).

Real-time RT-PCR

Total RNAs were isolated from LNCaP cells using a RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. One microgram of total RNAs was used to generate cDNA using an iScript cDNA synthesis kit. Real-time PCR was subsequently performed using SsoFast EvaGreen Supermix on a CFX96 Real-Time PCR Detection System (Bio-Rad, Richmond, CA, USA). The following primers were used: PKD1, forward primer 5′-CGCACATCATCTG CTGAACT-3′ and reverse primer 5′-CTTTCGGTGCA

CAACGTTTA-3′; FRS2, forward primer 5′-ATGG GAATGAGTTAGGTTCTGGC-3′ and reverse primer 5′-GCGGGTGTATAAAATCAGTTCTGTG-3′. Data were normalized automatically by using GAPDH as the loading control, with the following primers: forward primer 5′-GCAAATTCCATGGCACCGT-3′ and reverse primer 5′-TCGCCCCACTTGATTTTGG-3′.

Statistical analysis

All statistical analyse were carried out using GraphPad Prism IV software. A p value of less than 0.05 was considered statistically significant ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$).

ACKNOWLEDGMENTS

We would like to thank Pat Fawcett, Ravi Patel, and Dr. Karthik Giridhar for technical assistance on this project.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

GRANT SUPPORT

This study was supported in part by the National Institutes of Health grant R01CA129127 (Wang), R01CA142580 (Wang), Department of Defense award PC150190, and Oversea Hong Kong & Macao Scholars Collaborative Research Fund of NSFC in China (Grant# 81328020, Deng and Wang).

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Published OnlineFirst July 17, 2018; DOI: 10.1158/1541-7786.MCR-18-0641

Signal Transduction

Molecular Cancer Research

Protein Kinase D2 Modulates Cell Cycle By Stabilizing Aurora A Kinase at Centrosomes

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Abstract

Aurora A kinase (AURKA) is a master cell-cycle regulator that is often dysregulated in human cancers. Its overexpression has been associated with genome instability and oncogenic transformation. The protein kinase D (PKD) family is an emerging therapeutic target of cancer. Aberrant PKD activation has been implicated in tumor growth and survival, yet the underlying mechanisms remain to be elucidated. This study identified, for the first time, a functional crosstalk between PKD2 and Aurora A kinase in cancer cells. The data demonstrate that PKD2 is catalytically active during the G_2–M phases of the cell cycle, and inactivation or depletion of PKD2 causes delay in mitotic entry due to downregulation of Aurora A, an effect that can be rescued by overexpression of Aurora A. Moreover, PKD2 localizes in the centrosome with Aurora A by binding to γ-tubulin. Knockdown of PKD2 caused defects in centrosome separation, elongated G_2 phase, mitotic catastrophe, and eventually cell death via apoptosis. Mechanistically, PKD2 interferes with Fbxw7 function to protect Aurora A from ubiquitin- and proteasome-dependent degradation. Taken together, these results identify PKD as a cell-cycle checkpoint kinase that positively modulates G_2–M transition through Aurora A kinase in mammalian cells.

Implications: PKD2 is a novel cell-cycle regulator that promotes G_2–M transition by modulating Aurora A kinase stability in cancer cells and suggests the PKD2/Aurora A kinase regulatory axis as new therapeutic targets for cancer treatment.

Mol Cancer Res; 1–13. ©2018 AACR.

Introduction

Cell cycle is propelled by well-coordinated complex signaling events by which cells grow and divide. Uncontrolled cell division, whereby cells go through the cycle unchecked, lies at the base of cancer formation and progression. Cell cycle occurs through progression of four distinct stages, namely G_0–G_1, S, G_2, and M, which are monitored by a battery of cyclin-dependent kinases (CDK) and their partner cyclins (1). Mutations in the signaling pathways, aberrant activation of CDKs, genetic lesions in the genes encoding cell-cycle–regulatory proteins result in genome instability, abnormal growth, and eventually cancer.

The protein kinase D (PKD) family of serine/threonine kinases belongs to the Ca^{++}/Calmodulin-dependent protein kinase (CaMK) superfamily and it consists of three isoforms in mammals, notably, PKD1, PKD2, and PKD3, which are highly conserved throughout evolution (2). Structurally, PKD possesses an N-terminal regulatory domain that contains a tandem cysteine-rich Zn-finger like motif (CRD, C1a, and C1b) and a plekstrin homology (PH) domain, and a C-terminal catalytic domain (3, 4). In a canonical pathway, diverse physiologic factors, such as GPCR agonists, bioactive peptides (5), lipids (6), and growth factors (7) converge to the activation of PKDs through the generation of diacylglycerol (DAG) by phospholipase C (PLC) and the activation of classical or novel protein kinase C (c/nPKC; ref. 8). Activation of PKD is marked by phosphorylation of two conserved serine residues in the activation loop of PKDs and concomitant autophosphorylation of PKD1^{Ser916} or PKD2^{Ser876} are widely used as a biomarker of PKD activation (3, 9). PKD regulates a wide range of cellular processes including cell proliferation, migration/invasion, angiogenesis, protein trafficking, and gene expression. Its functional importance has been implicated in major human cancers including carcinomas of breast, skin, pancreas, and prostate (8).

Aurora kinases are master regulators of the cell cycle (10–13). Human genome encodes three isoforms of Aurora kinases; namely, Aurora kinases A, B, and C (14). Aurora A localizes on centrosomes, spindle poles, and spindles during mitosis and regulates centrosome function, spindle assembly, and mitotic progression. Expression of Aurora A is cell-cycle–regulated, that is, the levels of mRNA and protein are low in G_1 and S, increase during G_2–M, and reduce during mitotic exit. Aurora A is ubiquitinated by APC/Cdh1 and Fbxw7 ubiquitin ligases and degraded in proteasome-dependent pathways (15, 16). A number of Aurora A substrates have been reported, such as TPX2, CDC25B, and p53 (17). Aurora A is frequently overexpressed in cancer. Aberrant expression of Aurora A promotes centrosome amplification and aneuploidy, leading to genome instability and consequently oncogenic transformation (18). Thus, Aurora A represents a well-established therapeutic target for cancer. In this study, we uncovered a novel crosstalk between PKD2 and Aurora A kinase. Our study defined the functional impact of this regulatory axis on G_2–M transition and mitosis. We have shown that PKD2 is required for stabilization of Aurora A kinase, and that the abrogation of PKD2 expression or inhibition of its catalytic activity causes proteasome-mediated downregulation of Aurora A kinase displaying delayed G_2, mitotic catastrophe and cell death. Moreover, we report that

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doi: 10.1158/1541-7786.MCR-18-0641

Published OnlineFirst July 17, 2018; DOI: 10.1158/1541-7786.MCR-18-0641

PKD2 localizes on the centrosomes during G_2 and that it is critical for centrosome separation in early G_2. Taken together, our study provides compelling evidence to support the role of PKD2 in G_2–M transition through modulating Aurora A stability, and aberrant PKD2 activity may contribute to oncogenesis.

Materials and Methods

Cell culture, synchronization, and treatments

Authenticated HeLa, LNCap, and PC3 cells were obtained from ATCC. After purchase, the cell lines were expanded and frozen according to manufacturer's instructions after two to three passages. LNCaP was used for no more than 8 passages and PC3/HeLa cells were used for no more than 10 passages. All cell cultures were routinely tested for *Mycoplasma* using MycoAlert PLUS Mycoplasma Detection Kit (Lonza). HeLa, LNCaP, and PC3 cells were grown in 1× Minimum Essential Medium (MEM) supplemented with Eagle salt and L-glutamine (Invitrogen), RPMI1640 (Invitrogen), or Ham F-12 (Thermo Fisher Scientific) media, respectively, supplemented with 10% FBS and 1× penicillin/streptomycin (Thermo Fisher Scientific, MT30002CI) and maintained at 37°C in a humidified incubator containing 5% CO_2. Cells were synchronized at G_1–S or the start of M phases by double thymidine or nocodazole, respectively. Briefly, for G_1–S synchronization, cells were treated with 2 mmol/L thymidine for 16 hours, washed three times with 1× PBS, and refed into fresh culture medium without thymidine for 8 hours. The second block was performed by treating the cells with 2 mmol/L thymidine for another 16 hours. Cell arrest at the start of M phase was performed by treating the cells with nocodazole (100 ng/mL) for 16 hours. PKD inhibitors (CRT0066101 and kb-NB142-70) were used at a final concentration of 2 μmol/L for indicated times. Turbofect (Thermo Fisher Scientific) and Lipofectamine 3000 (Invitrogen) transfection reagents were used to transfect plasmids and siRNAs, respectively, according to the manufacturer's protocol.

Cell survival assay

Cell survival assay was performed by Cell Counting Kit-8 (Dojindo Laboratories) according to the manufacturer's protocol. Briefly, HeLa and PC3 cells were plated at a density of 4,000 cells per well in 96-well plate. After treating the cells with different doses of drugs (CRT0066101 and Kb-NB142-70) for 72 hours, CCK-8 solution was added to cells in each well, followed by incubation for 2 hours. Cell proliferation/viability was determined by measuring the OD at 450 nm.

Western blotting and densitometry analysis

Cells were lysed in cell lysis buffer (50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 10% glycerol, 1% Triton X-100, 5 mmol/L EGTA, 1 mmol/L Na_3VO_4, 10 mmol/L NaF, 1 mmol/L β-glycerophosphate and protease inhibitor cocktail). Cell extracts were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with following antibodies: PKD2 (Cell Signaling Technology, 8188), phospho-PKD2^{Ser876} (Thermo Fisher Scientific, PA5-64538), Cyclin B1 (Cell Signaling Technology, 12231), phospho CDC25^{Thr48} (Cell Signaling Technology 12028), phospho-Histone H3^{Ser10} (Cell Signaling Technology, 3377), Aurora Kinase A (Cell Signaling Technology, 14475), Aurora Kinase B (Cell Signaling Technology, 3094), γ-tubulin (Santa Cruz Biotechnology, sc-17788), PARP (Cell Signaling Technology, 9542), ubiquitin (P4D1, Cell Signaling Technology 3936), Lamin A (Cell Signaling Technology, 2032), Cdh1 (Santa Cruz Biotechnology, sc-56312), Fbxw7 (Abcam, EPR8069), and GAPDH (Enzo ADI-CSA-335-E). Protein half-life was measured as described previously (19). Briefly, the band intensities were quantified by ImageJ software and the values of target proteins were normalized against the band intensity of GAPDH. The measured protein intensity values were log transformed, fitted to a linear regression plot, and decay rate constant (k) was determined. The half-life was calculated from k using the formula $T\frac{1}{2} = \ln(2)/k$.

Immunoprecipitation analysis

For immunoprecipitation assay, whole-cell extract was prepared by lysing the cells with cell lysis buffer. The lysate was centrifuged, supernatant was collected, and protein concentration was estimated using BCA protein assay kit (Pierce) according to manufacturer's protocol. Cell lysate containing equal amount of total protein was incubated with primary antibodies at 4°C overnight. Protein A/G agarose beads were added later for another 2 hours. The immunoprecipitates were collected and washed with wash buffer containing 0.5% Triton X-100. Finally, the samples were diluted with 6× SDS PAGE loading buffer, boiled for 10 minutes, and subjected to Western blotting.

Subcellular fractionation

Cells were washed with ice-cold PBS, collected by centrifugation at 1,000 rpm for 5 minutes, and resuspended in cytoplasm extraction buffer (10 mmol/L HEPES pH 7.5, 60 mmol/L KCl, 1 mmol/L EDTA, 0.1% NP-40, 1 mmol/L DTT, and protease inhibitor cocktail) for 5 minutes on ice. The cell lysate was centrifuged at 1,000 rpm for 5 minutes at 4°C and cytoplasmic fraction was separated in a new tube. The nuclei were washed in cytoplasm extraction buffer without NP-40 and resuspended in nucleus extraction buffer (20 mmol/L Tris-HCl pH 8, 400 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 25% glycerol and protease inhibitor cocktail). The extracts were incubated on ice for 10 minutes. Both cytoplasmic and nuclear extracts were centrifuged at 14,000 rpm for 15 minutes at 4°C and transferred to new tubes.

Flow cytometry

Trypsinized cells were pelleted, washed two times with ice-cold FACS buffer (0.1% glucose in PBS), and resuspended in ice cold 70% ethanol while vortexing. Cells were fixed by incubating overnight at 4°C, washed with PBS, and incubated with 1 mL propidium iodide (PI) staining solution (50 μg/mL PI, 0.1 mg/mL RNase A in FACS buffer) for 30 minutes. Cells were analyzed in a FACSCalibur (BD Biosciences) flow cytometer and the data were analyzed by ModFit 3.0 software (BD Biosciences). A total of 10,000 events were analyzed for each sample.

Apoptosis assay

Trypsinized cells were washed twice with ice-cold PBS and stained with Annexin V-Alexa Fluor 488 antibody and propidium iodide (Molecular Probes, Thermo Fisher Scientific) according to the manufacturer's instructions. The samples were divided in two parts. One half was subjected to FACSCalibur (BD Biosciences) flow cytometer and the other part was subjected to confocal fluorescence microscopy using Alexa Fluor 488 and PI filters.

Published OnlineFirst July 17, 2018; DOI: 10.1158/1541-7786.MCR-18-0641

Immunofluorescence staining and microscopy

HeLa and PC3 cells were grown on poly-D-lysine–coated coverslips, washed with PBS, fixed in 4% paraformaldehyde at room temperature for 30 minutes, washed three times with 1× PBS, and blocked in 5% normal goat serum containing 0.3% Triton X-100 for 1 hour at room temperature. The cells were incubated with the primary antibodies diluted in antibody dilution buffer (1% BSA, 0.3% Triton X-100 in 1× PBS) overnight at 4°C, washed three times in 1× PBS, and incubated with secondary antibodies diluted in antibody dilution buffer for 1 hour at room temperature. The cells were counterstained with DAPI (1 μg/mL) and the coverslips were mounted in ProLong Gold antifade reagent (Invitrogen). The cells were analyzed using an Olympus Fluoview (FV1000) confocal microscope using 60 X/1.45 objectives.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Statistical analysis

Data analysis was done using the Student *t* test for comparison between two groups (two-tailed). All statistical analyses were done using GraphPad Prism IV software (GraphPad Software). All values are represented as mean ± SEM of at least three independent experiments. A $P < 0.05$ was considered statistically significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant).

Results

Elevated PKD2 activity during mitotic entry of the cell cycle

First, we examined the expression and activity of PKD (refers to PKD2 and PKD3 throughout the text) in different cell-cycle stages. PKD2 and PKD3 are major isoforms expressed in HeLa cells (Supplementary Fig. S1), similar to the prostate cancer cell line PC3, but differed from LNCaP cells (20). HeLa and PC3 cells were treated with nocodazole, a microtubule-destabilizing agent that activates the spindle assembly checkpoint and causes cell arrest in start of M-phase (prometaphase), and the activity of PKD2 was assessed after releasing from nocodazole using the phospho-PKD2^{Ser876} (p-PKD2) antibody. As shown in Fig. 1A (top) and B, p-PKD2 was detected in G_2–M–arrested HeLa cells following nocodazole synchronization and persisted till the end of mitosis. This was accompanied by increased cyclin B1, a marker for G_2 and M phases of the cell cycle, and phospho-Histone H3^{Ser10} (pHH3), which was detectable in G_2, peaks during mitosis and disappears when cells enters in G_1 (Fig. 1A; Supplementary Fig. S2). Similar results were obtained with PC3 cells where activation of PKD2 was marked by increased levels of p-PKD2 after release from nocodazole arrest (Fig. 1A, bottom). To determine whether the activation is specific to G_2–M, PKD2 activation was measured in G_1–S synchronized HeLa cells that progressed to M-phase. As shown in Fig. 1C and D, when cells were released from a double thymidine (DT) block that synchronizes cells at G_1–S border, PKD2 phosphorylation gradually elevated, starting at 4 hours when cells entered in late S-phase peaked around 10 hours in late G_2, and persisted throughout mitosis till 14 hours after thymidine release. The highest activity was detected at G_2–M border and M-phase. This pattern correlated well with increased cyclin B1 and pHH3. As controls, PKD2 remained the same under both conditions.

The intracellular distribution of PKD2 was then analyzed at different stages of the cell cycle by immunofluorescence (IF) staining. In HeLa cells, PKD2 was primarily localized in the cytoplasm and perinuclear zone in interphase. When cells progress through cell cycle, it exhibited scattered punctate staining that are minimally overlapped with mitotic bodies (spindle fibers and midbody) and excluded from condensed chromatin (Fig. 1E, top). PKD2 phosphorylation was low or undetectable during interphase and significantly increased during G_2, sustained throughout prophase, metaphase, and anaphase, before returning to baseline when the cells entered telophase. To corroborate these results in cancer, PC3 cells were stained in similar fashion. Similar results were found when different stages of the cell cycle in PC3 cells were imaged using antibodies against phospho-PKD2^{Ser876} (green) and α-tubulin (red; Fig. 1F). It is well documented that PKD, upon activation, accumulates in the nucleus (21). Indeed, we observed elevated active PKD in the nucleus during G_2 and early prophase when the nucleus was still intact, further attesting that PKD2 was activated during G_2 and mitosis. We further validated that phospho-PKD2 was increased in the nucleus during early mitosis by cell fractionation (Fig. 1G, left). HeLa and PC3 cells were arrested at the start of M phase by nocodazole and cytoplasmic (Cyto) or nuclear (Nuc) fractions were prepared. The samples were analyzed by Western blotting using phospho-PKD2 antibody and the results showed that, indeed, active PKD2 levels were elevated in early mitosis (Fig. 1G, right). Lamin A was blotted as a marker for the nuclear fraction. Taken together, these results suggested that PKD is active during G_2 and mitosis and may play an important role in cell-cycle regulation.

Inactivation or knockdown of PKD2 causes delay in G_2–M transition

The activation profile of PKD2 suggests that PKD2 may regulate G_2 and M-phase cell-cycle progression. To test this hypothesis, cells with altered PKD2 expression and activity were synchronized by DT at G_1–S border and released (Figs. 2A and 3A); cell-cycle progression was analyzed by flow cytometry. As shown in Fig. 2B and C, after thymidine release, cells reached G_2–M at 8 hours, and by 12 hours, cells resumed normal distribution. In contrast, cells treated with the PKD inhibitor CRT0066101 (CRT101) reached G_2–M at 8 hours, stayed after 12 hours, and by 14 hours there remained a large G_2–M population as compared with the control. Inactivation of PKD by another PKD inhibitor kb-NB142-70 (kb-NB) gave rise to similar results (Fig. 2B, kb-NB). Quantification analysis confirmed prolonged G_2–M phase of cell cycle in cells treated with PKC inhibitors (Fig. 2C). Thus, inactivation PKD2 prolonged G_2–M phase and resulted in delayed mitotic entry in cells.

To determine that PKD2 activity was indeed required for G_2–M transition, cells transfected with PKD2 siRNAs were synchronized to G_1–S border by DT and released; cell-cycle progression was monitored by flow cytometry (Fig. 3A). Our data showed that cells transfected with nontargeting siRNA reached G_2–M around 8 hours and returned to normal distribution at about 12–14 hours. In contrast, cells transfected with two PKD2 siRNAs (Fig. 3B) progressed slower through the cell cycle and peaked at G_2–M phase around 12 hours and remained till after 14 hours (Fig. 3C), indicating that PKD2-depleted cells progressed through G_2–M boundary at much slower rate than control cells. Knockdown of PKD2 using two siRNAs in these samples were confirmed by Western blotting (Fig. 3D). To corroborate these findings, we

Published OnlineFirst July 17, 2018; DOI: 10.1158/1541-7786.MCR-18-0641

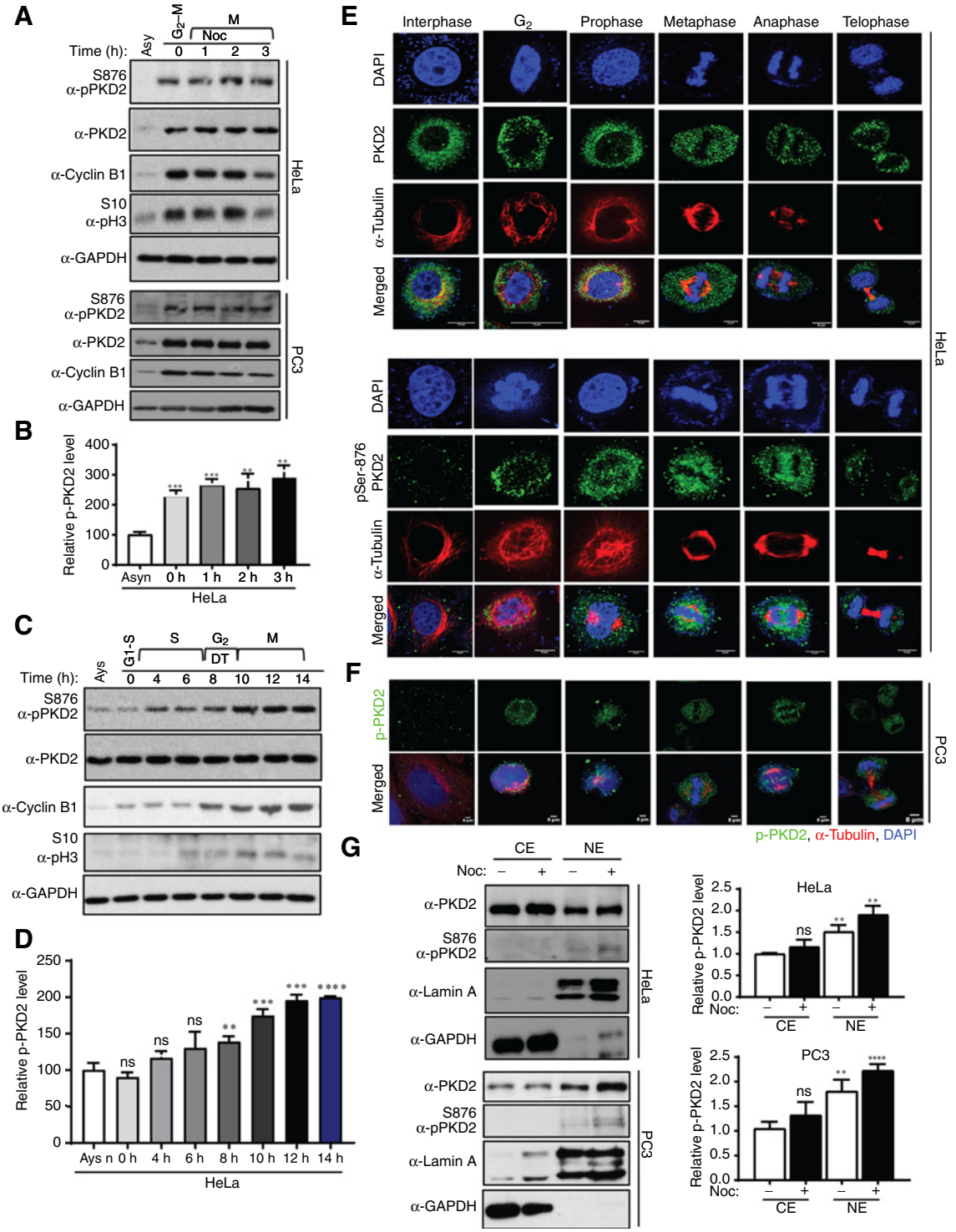

Figure 1.

PKD is activated during mitotic entry. **A,** HeLa and PC3 cells were synchronized with nocodazole (100 ng/mL) for 16 hours and mitotic cells were released into fresh medium for indicated times. Cell extracts from each sample were subjected to Western blot analysis to analyze activation of PKD2. **B,** Densitometric quantification of phospho-PKD2^{S876} from the immunoblot of HeLa was performed. **C,** Western blot analysis of indicated proteins after the HeLa cells were released from a double thymidine (DT) block was performed. **D,** Densitometric quantification of phospho-PKD2^{S876} from the immunoblot of **C** was performed. Different phases of cell cycle are marked (G_1, S, G_2, and M). **E,** HeLa cells were subjected to immunofluorescence (IF) staining to visualize colocalization PKD2 or phospho-PKD2^{S876} with α-tubulin during different phases of cell cycle. **F,** PC3 cells were subjected to IF staining to visualize colocalization of phospho-PKD2^{S876} with α-tubulin during different phases of cell cycle. DAPI was used to stain the nucleus. Scale bar, 8 μm. GAPDH was used as loading control in **A** and **C**. **G,** HeLa and PC3 cells were synchronized with nocodazole (100 ng/mL) for 16 hours and mitotic cells were collected by shake-off. Cytoplasmic (Cyto) and nuclear (Nuc) fractions were prepared and the samples were analyzed by Western blotting with indicated antibodies (left). Relative amount of phospho-PKD2^{S876} was quantified (right). The graphs show average of three independent experiments with error bars representing SEM (**, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant).

Published OnlineFirst July 17, 2018; DOI: 10.1158/1541-7786.MCR-18-0641

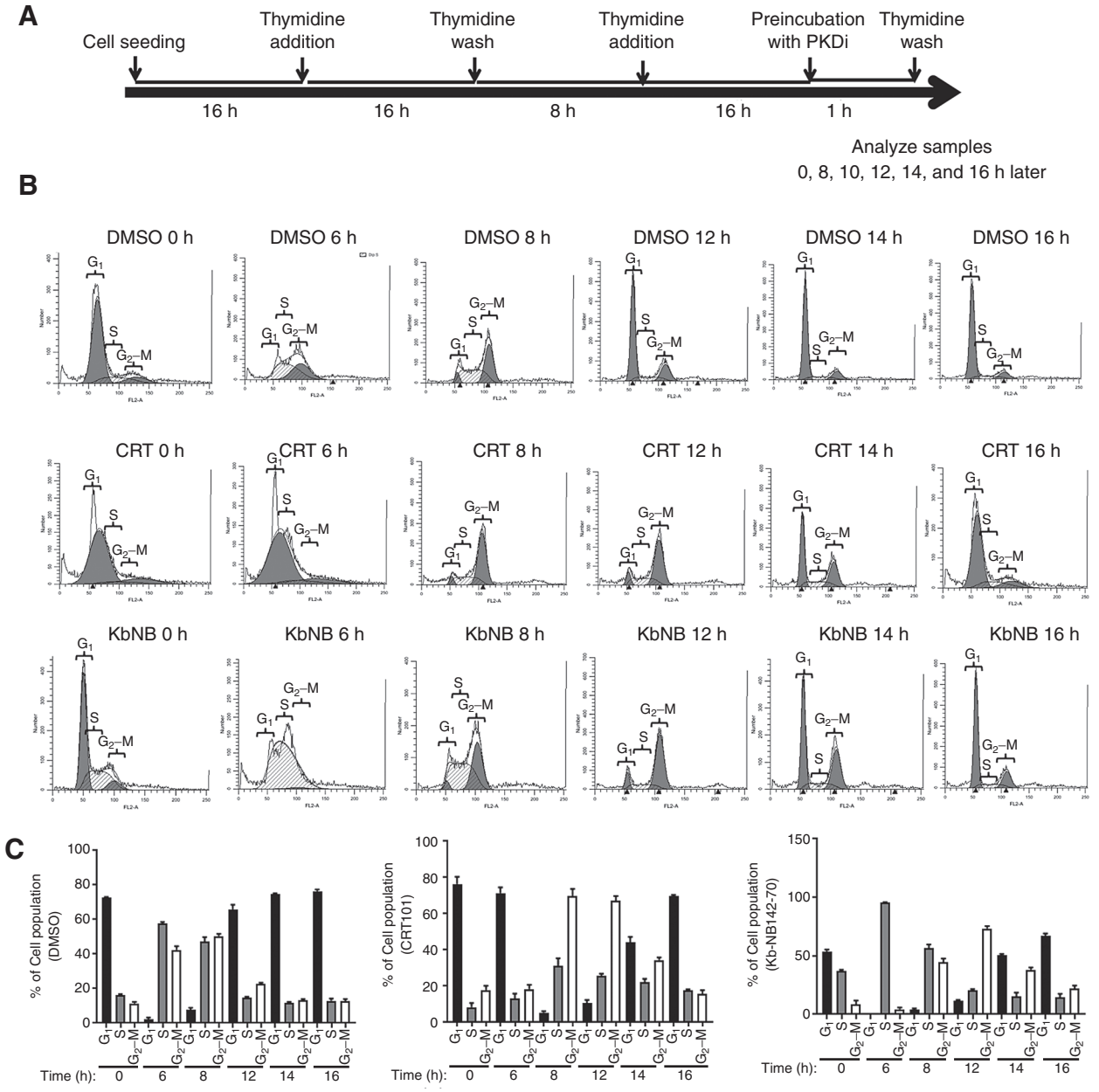

Figure 2.

Inactivation of PKD by CRT101 and kb-NB induces a delay in G_2–M progression. **A,** Schematic representation of experimental design is shown. **B,** Flow cytometry analysis of cells treated with DMSO, CRT101, and kb-NB were performed according to **A**. **C,** Summarizing graph of the distribution of different phases of cell cycle are shown (right). The graphs show average of three independent experiments with error bars representing SEM.

examined HeLa cells with overexpressed PKD2. As shown in Fig. 3E, overexpression of PKD2 significantly increased the number of cells entering mitosis. Taken together, our data indicate that PKD2 was activated in G_2–M and its activity was required for mitotic entry.

Abrogation of PKD2 expression sensitizes cells to mitotic catastrophe and cell death

Cells experience prolonged G_2 arrest and delayed mitotic entry may eventually enter into mitosis, however, they often suffer from aberrant mitosis known as mitotic catastrophe, and die subsequently by apoptosis (22). Here, we examined whether mitotic catastrophe was associated with loss/inactivation of PKD2 and delayed G_2–M transition. Knockdown or inactivation of PKD2 using two siRNAs or PKD inhibitor CRT101, respectively, induced a series of mitotic defects including chromosomal misalignment at metaphase plane, abnormal chromosomal arrangement (Fig. 4A, white arrows), and formation of multiple centrosomes (Fig. 4A, red arrows). Quantification analysis indicated that depletion or inactivation of PKD2 significantly increased cell

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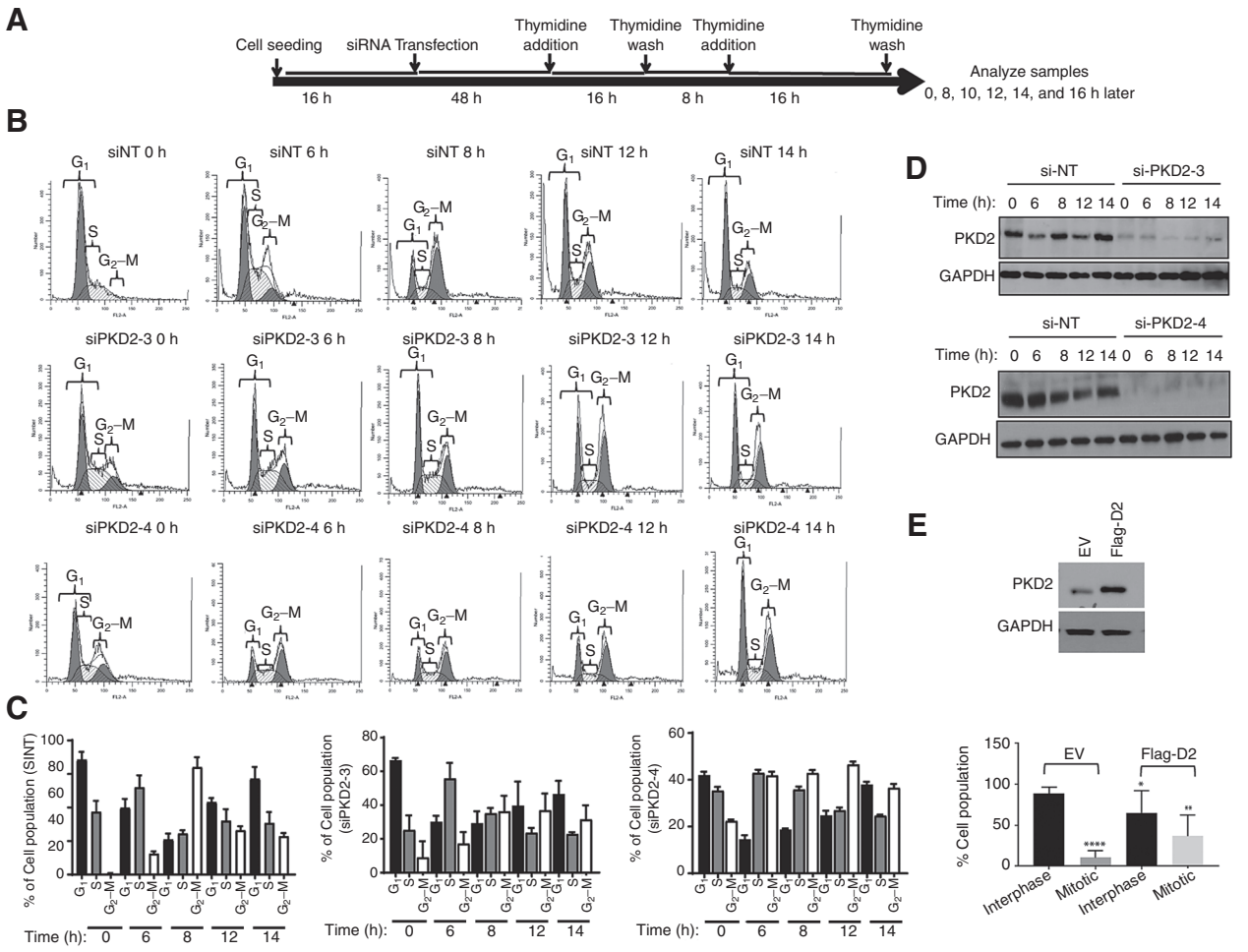

Figure 3.

Abrogation of PKD2 expression delays G_2–M progression. **A,** Schematic representation of experimental design is shown. **B,** Flow cytometry analysis of cells transfected with control nontargeting siRNA (siNT) and two siRNAs against PKD2 was performed according to **A. C,** Summarizing graph of the distribution of different phases of cell cycle are shown. The graphs show average of three independent experiments with error bars representing SEM. **D,** Western blot analysis of the samples from **A** was performed to confirm knockdown of PKD2. GAPDH was used as loading control. **E,** PKD2 was ectopically overexpressed using a plasmid expressing Flag-PKD2 (top) and percentage of interphase and mitotic cell population was counted (bottom). The graphs show average of three independent experiments with error bars representing SEM (*, $P < 0.5$; **, $P < 0.01$; ****, $P < 0.0001$).

population with defective mitosis (Fig. 4B). It is well known that mitotic catastrophe leads to apoptosis followed by PARP and Lamin A cleavage (23, 24). Analysis of PKD2-depleted or PKD-inactivated HeLa cells showed expression of cleaved $PARP^{Asp214}$ and Lamin A^{Asp230}, indicative of apoptotic response (Fig. 4C). To further this analysis, cells transfected with siNT and siPKD2s were stained with Annexin V-Alexa Fluor 488/propidium iodide (PI) and analyzed by flow cytometry (Fig. 4D, left). Knockdown of PKD2 caused significant cell death (PI-positive) with elevated apoptotic population (Annexin V position) as compared with the control samples (siNT; Fig. 4D, right). Treatment of both HeLa and PC3 cells using increasing concentrations of CRT101 and kb-NB reduced cell viability in a concentration-dependent manner (Fig. 4E). Taken together, our data indicated that G_2–M deficiencies caused by PKD2 depletion or inhibition could result in mitotic catastrophe and cell death followed by apoptosis.

Inactivation of PKD2 causes degradation of Aurora A, an effect that can be suppressed by overexpression of Aurora A

We showed that knockdown or inactivation of PKD2 caused prolonged G_2–M transition, delay in mitotic entry, and mitotic catastrophe (Figs. 2–4). In the order to gain insights into the role of PKD in cell cycle, we sought to identify its downstream targets. Through a whole-genome RNA-seq analysis conducted on cancer cells treated with CRT101 and kb-NB, we identified AURKA/Aurora A kinase as one of the top candidate genes altered by inhibition of PKD. Aurora A is a master regulator of G_2–M and mitosis (12, 25–27). First, using normal prostate epithelial cell line RWPE-1 and prostate adenocarcinoma (PAC) cell lines, we sought to identify whether expression level of PKD2 correlates with that of Aurora A kinase. As shown in Fig. 5A, we observed positive correlation between PKD2 and Aurora A in these cell lines. Next, we subjected HeLa cells to IF staining and checked whether PKD2 and Aurora A colocalize at different stages of

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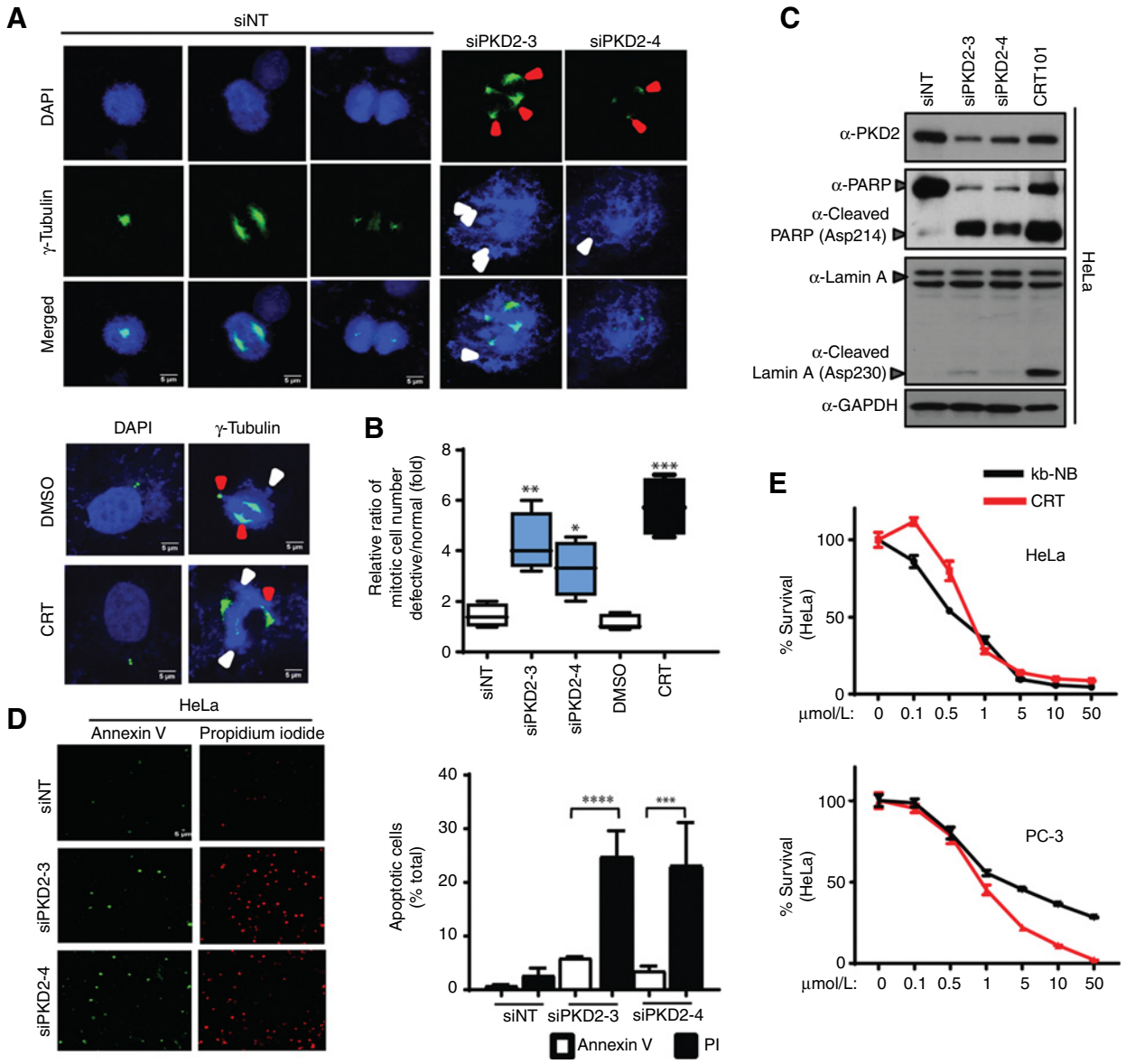

Figure 4.

Knockdown of PKD2 causes mitotic catastrophe followed by cell death. **A,** IF staining of cells transfected with control nontargeting siRNA (siNT) and two siRNAs against PKD2 (top) or DMSO/CRT101 (bottom) was performed to visualize γ-tubulin (green). DAPI was used to stain the nucleus. Red and white arrow heads indicate defects in spindle pole formation and misaligned chromosomes, respectively. **B,** Quantification of cell population harboring mitotic defects after the cells were treated with either si-PKD2 (top) or CRT101 (bottom). **C,** Western blot analysis was done to show cleavage of PARP/Lamin A upon depletion or inactivation of PKD2 in HeLa. GAPDH served as loading control. **D,** Microscopic analysis of cells transfected with siNT or two siPKD2s after costaining with Annexin V-Alexa Fluor 488 and propidium iodide (left). Quantification of cell population positive for Annexin V-Alexa Fluor 488 and PI (right). **E,** HeLa and PC3 cells were treated with different concentrations of PKD inhibitors (CRT101, kb-NB) and cultured for 72 hours. MTT assay was performed to assess cell viability. The graphs show average of three independent experiments with error bars representing SEM (***, $P < 0.001$; ****, $P < 0.0001$).

mitosis. Our confocal microscopy results showed that a significant portion of PKD2 (green) colocalizes with Aurora A (red) during mitosis (Fig. 5B), raising a strong possibility that PKD2 might contribute to cell cycle by regulating Aurora A. We examined the expression levels of Aurora A upon inactivation or depletion of PKD. HeLa cells were arrested at the start of M phase by nocodazole, released from the block by mitotic shake off and turnover of Aurora A protein levels was analyzed in the absence or presence of CRT101 by Western blotting. The results showed that CRT101 caused rapid downregulation of Aurora A with < 50% remaining 2 hours after CRT101 treatment (Fig. 5C). To corroborate this finding in the context of cancer, we treated PC3 prostate adenocarcinoma and COV362 ovarian epithelial-endometroid carcinoma cell lines as described above and checked Aurora A turnover by Western blotting (Fig. 5D). As expected, Aurora A was rapidly downregulated by CRT101 in these two cell lines.

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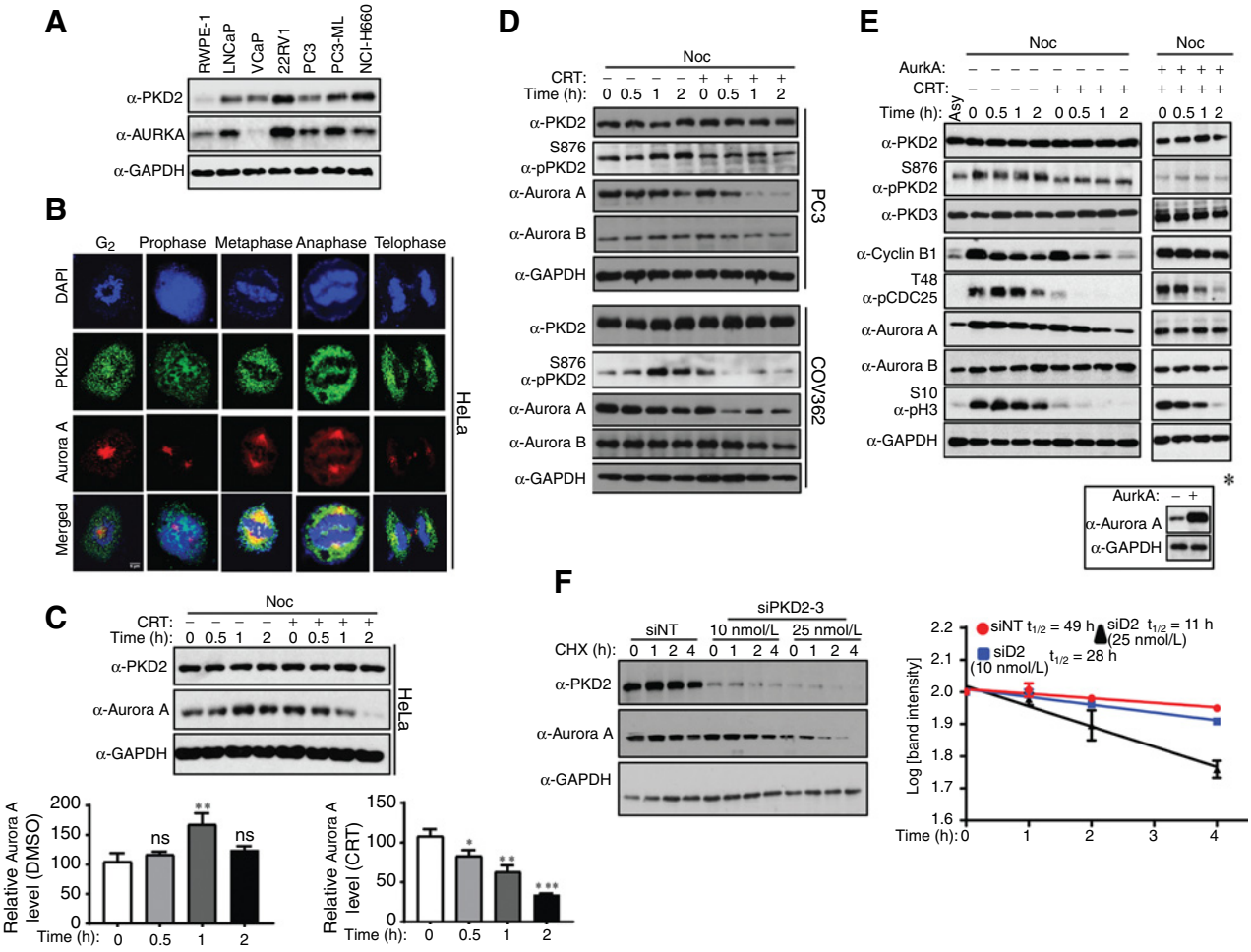

Figure 5.

Inactivation or knockdown of PKD2 causes downregulation of Aurora A kinase. **A,** Western blot analysis was performed to determine expression patterns of PKD2 and Aurora A in designated cell lines. **B,** HeLa cells were stained with antibodies against PKD2 (green) and Aurora A (red). IF staining was performed to visualize colocalization of PKD2 and Aurora A at different stages of cell cycle. Scale bar, 8 μm. DAPI was used to stain the nucleus. **C,** HeLa cells were synchronized at the start of M-phase by nocodazole, released from the block and mitotic cells were cultured for indicated times with or without CRT101. Downregulation of Aurora A was analyzed by Western blotting (top). Quantification of Aurora A protein levels by densitometry scanning from immunoblots was performed (bottom). **D,** PC3 and COV362 cells were treated as described in **C** and turnover of Aurora A protein levels were analyzed by Western blotting. The expression levels of Aurora B were analyzed as control. **E,** HeLa Cells were arrested at the start of M-phase by nocodazole (noc), released in fresh medium containing DMSO or CRT101 for indicated times and cell extracts from each samples were analyzed by Western blotting using indicated antibodies (left). Aurora A was ectopically overexpressed and the cells were released from nocodazole block in fresh medium containing CRT101 for indicated times. Cell extracts from each sample were analyzed by Western blotting using indicated antibodies. Overexpression of Aurora A was confirmed by Western blotting. **F,** Cells were transfected by si-NT (control) and two different concentrations of si-PKD2. Forty-eight hours post transfection, cells were treated with cycloheximide for indicated times and cell extracts from each sample were analyzed by Western blotting using indicated antibodies (left). GAPDH served as loading control. Half-life of Aurora A protein was measured from the samples of **F** (right). The graphs show average of three independent experiments with error bars representing SEM (*, $P < 0.5$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant).

Meanwhile, the downregulation of Aurora A coupled with time-dependent decline of cyclin B1, p-CDC25^{T48} and p-HH3, which are in line with a critical role of PKD activity in G_2–M transition and mitotic progression (Fig. 5E, left). In comparison, the expression levels of Aurora B did not significantly change (Fig. 5D and E, Aurora B), indicating a selective effect of CRT101 on Aurora A degradation. Importantly, when ectopically overexpressed, Aurora A completely reversed the effect of CRT101 on cell-cycle markers (Fig. 5E, right; Supplementary Fig. S3). Thus, Aurora A mediated the effect of PKD inhibition on G_2–M transition. To corroborate these findings, Aurora A protein stability was assessed in cells transfected with siPKD2-3 (siD2) and treated with cycloheximide (CHX) that blocks protein synthesis. In the control cells (siNT), the half-life ($t_{1/2}$) of Aurora A was 49 hours. Partial knockdown of PKD2 reduced $t_{1/2}$ of Aurora A to about 28 hours, while nearly complete knockdown further reduced it to 11 hours (Fig. 5F). Similarly, knockdown of PKD3 with two siRNAs also resulted in downregulation of Aurora A (Supplementary Fig. S5). These results identify Aurora A as a key mediator of PKD2-regulated cell-cycle progression, and the activation of PKD2 at G_2–M stabilizes Aurora A kinase and promotes mitotic entry.

Published OnlineFirst July 17, 2018; DOI: 10.1158/1541-7786.MCR-18-0641

PKD2 and Aurora A localize at the centrosome and knockdown of PKD2 inhibits centrosome separation during G_2

Aurora A has been shown to localize at mitotic apparatus and contribute to G_2–M transition and mitotic progression (13, 22, 28, 29). Aurora A resides in the centrosome and regulates centrosome duplication and separation, inhibition of Aurora A arrests cells in G2 and blocks mitotic entry. Here, we sought to determine whether the regulation of Aurora A by PKD also took place at the centrosome and whether it affects centrosome function. HeLa cells were costained with PKD2 and γ-tubulin, a marker of the centrosome. As shown in Fig. 6A, PKD2 (red) was found to colocalize with γ-tubulin (green) at different stages of cell cycle, and the colocalization was most prominent in the G_2 stage. Next, cells were synchronized to the start of M-phase by nocodazole and the interaction of PKD2 and γ-tubulin were examined by reciprocal immunoprecipitation. As shown in Fig. 6B, γ-tubulin was detected in immunoprecipitated PKD2 complex, and vice versa. Thus, a portion of PKD2 was present on the centrosome and PKD2 directly interacts with γ-tubulin at the centrosome during G_2–M, which was further confirmed by IF staining (Fig. 6C). Next, we examined whether PKD2 might directly interact with Aurora A. Aurora A is known to localize at the centrosome (13, 27, 30, 31). Our data confirmed that Aurora A (red) colocalized with γ-tubulin (green) during G_2 and mitosis (Supplementary Fig. S6). By costaining for PKD2 and Aurora A, we also found significant colocalization of PKD2 (green) and Aurora A (red) during G_2 and mitosis (Fig. 5A). Colocalization of PKD2 and Aurora A on the centrosome raised a possibility that they might physically interact. However, our follow-up study indicated that PKD2 failed to coimmunoprecipitate with Aurora A in G_2–M–synchronized cells (Supplementary Fig. S7). Despite the lack of direct interaction, the colocalization of PKD2 and Aurora A support the potential functional link between the two proteins in centrosome. Similar colocalization pattern was observed when the intracellular distributions of PKD3 and Aurora A were analyzed during G_2 and mitosis (Supplementary Fig. S7). Aurora A is crucial for

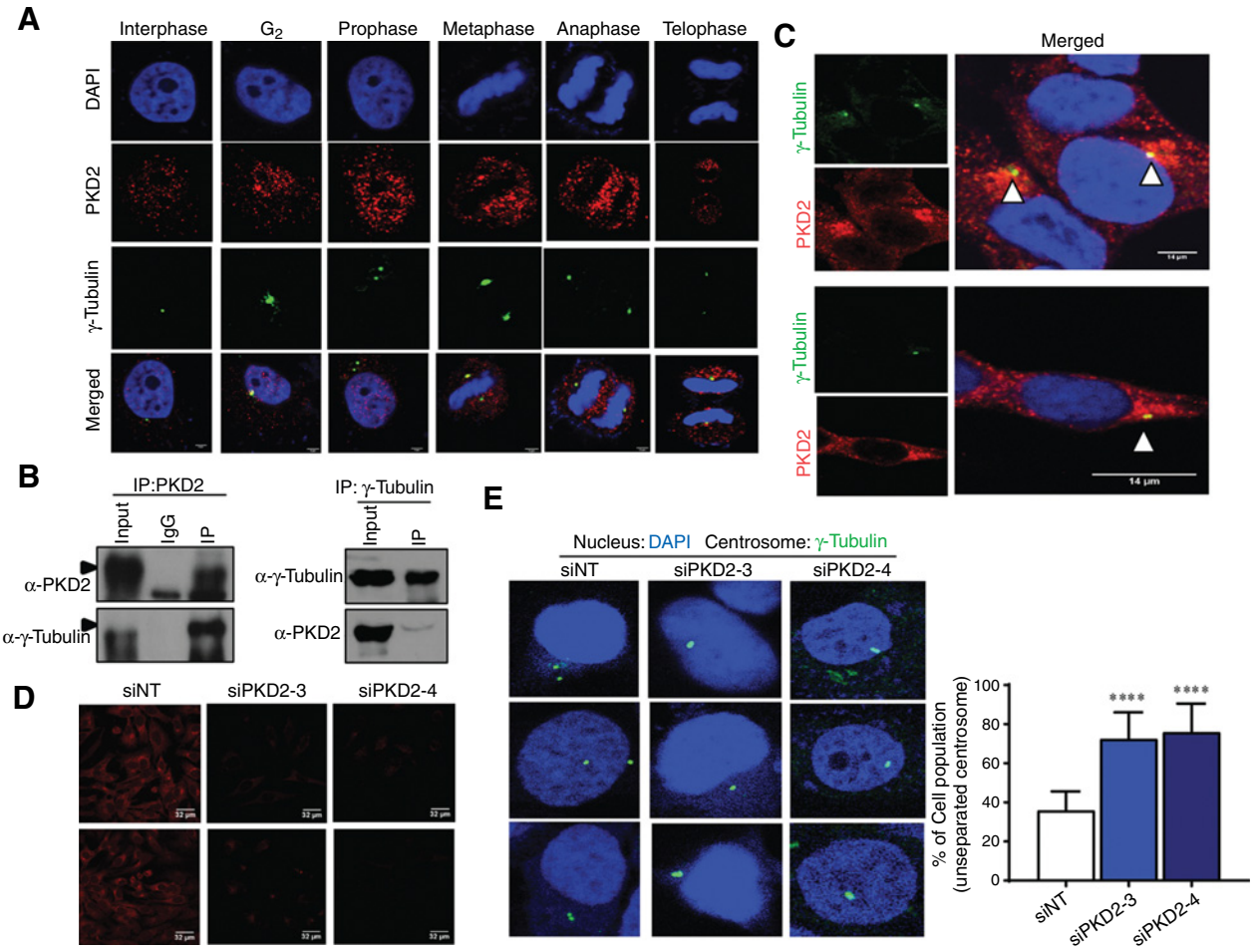

Figure 6.

PKD2 positively regulates centriole separation during late G_2 by colocalizing with centrosomes. **A–C,** HeLa cell was subjected to IF staining to visualize colocalization of PKD2 (red) and γ-tubulin (green). **B,** Coimmunoprecipitation assay was carried out to assess physical interaction between PKD2 and γ-tubulin. **D,** Knockdown of PKD2 by two siRNAs was confirmed by IF staining. **E,** Centrioles were visualized by IF staining of γ-tubulin in cells transfected with siNT or two siPKD2s (left). Cell population harboring unseparated centrioles was quantified (right). At least 100 cells were counted and graph shows average number of cells with error bars representing SEM (****, $P < 0.0001$). DAPI was used to stain the nucleus.

Published OnlineFirst July 17, 2018; DOI: 10.1158/1541-7786.MCR-18-0641

centrosome maturation and separation during G_2 (31). Hence, we questioned whether loss of Aurora A by depletion of PKD2 caused any defect in centrosome separation. To do so, cells were transfected with two siPKD2s followed by IF staining of γ-tubulin to visualize the centrioles. Knockdown of PKD2 was confirmed by IF staining (Fig. 6D, red). Our results showed that depletion of PKD2 resulted in defects in centriole separation (Fig. 6E, left) and significantly increased cell population harboring unseparated centrosomes (Fig. 6E, right). Taken together, these results suggest that PKD positively regulates stabilization of Aurora A and centrosome separation to promote G_2–M progression.

PKD2 interferes with Fbxw7-mediated ubiquitination and downregulation of Aurora A

To assess whether PKD regulates Aurora A degradation through the proteasome degradation pathway, cells were synchronized with nocodazole, released in fresh medium containing CRT101 in the presence or absence of a proteasome inhibitor MG132, and the levels of Aurora A were examined by immunoblotting. Our data showed that Aurora A levels were dampened within 3 hours of CRT101 treatment and MG132 completely inhibited CRT101-induced Aurora A degradation (Fig. 7A). The half-life of Aurora A was measured in the presence of cycloheximide (CHX). As shown in Fig. 7B, treatment of CRT101 reduced the half-life of Aurora A protein to about 5 hours, whereas MG132 restored its half-life like control sample (nocodazole only; Fig. 7B). These results demonstrated that CRT101 directed Aurora A to the proteasomal pathway for its degradation. To further corroborate this finding, G_2–M–arrested cells were released in medium containing MG132 with or without CRT101, and cell extracts from the samples were subjected to IP using anti-Aurora A antibody, followed by immunoblotting with anti-ubiquitin antibody (Fig. 7C). We found that CRT101 increased ubiquitination of Aurora A (Fig. 7C, top) confirming that Aurora A is downregulated via ubiquitination-dependent proteasomal degradation pathway upon treatment with CRT101. Aurora A is targeted by Cdh1 and F-box protein Fbxw7 (15, 32) for degradation. Therefore, to gain insight into PKD-mediated downregulation of Aurora A, we knocked down PKD2, Cdh1, and Fbxw7 using site-specific siRNAs and analyzed the turnover of Aurora A protein levels. Knockdown of PKD2 downregulated Aurora A, whereas depletion of Cdh1 did not significantly alter Aurora A protein level (Fig. 7D). Interestingly, when Fbxw7 was depleted in PKD2 knocked down cells, Aurora A level was significantly restored as compared with siPKD2 (Fig. 7E). To further corroborate our findings, cells were transfected with nontargeting siRNA (siNT) or siFbxw7, synchronized at the start of M-phase by nocodazole, treated with CRT101 and cell extracts were analyzed by Western blotting to monitor Aurora A degradation (Fig. 7F). The immunoblot showed that Aurora A was downregulated within 3 hours of CRT101 treatment and this turnover was inhibited by Fbxw7 knockdown. These results suggested that PKD protects Aurora A from Fbxw7-mediated ubiquitination and proteasomal degradation to regulate G_2–M transition.

Discussion

PKD as a key target of the second messenger DAG regulates many important cellular functions including cell proliferation. Aberrant expression and activation of PKD have been demonstrated in many cancers and are implicated in oncogenic transformation and tumor progression. PKD2 in particular has been shown to promote tumor cell proliferation in human cancer including carcinoma of the prostate (8). However, the mechanisms through which it regulates tumor cell proliferation have not been well defined. In this study, we identified PKD2 as a cell-cycle regulator that promotes G_2–M transition and mitotic entry. PKD2 is activated during G_2 and M phases of the cell cycle. It is localized at the centrosome and upon activation regulates centrosome duplication and separation, a key event for the cells to exit G_2 and enter mitosis. Mechanistically, we further demonstrated that PKD activity was required for Aurora A stability during G_2–M, and it acts through Fbxw7 to protect Aurora A from ubiquitination and proteasomal degradation. Our study has identified PKD2 as a potential G_2–M checkpoint kinase. Its aberrant activation may contribute to premature mitotic entry and genome instability that drive tumorigenesis.

A key finding from our study is that PKD is active during G_2 and its activity is sustained throughout mitosis (Fig. 1F). Increased PKD2 activity in G_2–M was supported by: (i) immunoblotting results showing elevated active form of PKD2 in cells entering G_2 and progressing through mitosis (Fig. 1A–D), (ii) IF staining results showing increased active PKD2 staining in cell nuclei during G_2 (Fig. 1) and prophase of mitosis, indicative of PKD activation (21). Our finding that activation of PKD is crucial for G_2–M cell-cycle progression agrees with the report by Papazyan and colleagues that demonstrated a possible connection of PKD activation during mitosis and its role in cell cycle (33). The effects of PKD inhibitors are in line with previous reports of anticancer effects of PKDis. CRT0066101 (CRT101) in particular has been shown to block cell proliferation by inducing G_2–M arrest, downregulation of mitotic regulatory proteins and induction of apoptosis in colorectal (34), bladder (35), pancreatic (36), and prostate (37) cancer cell lines. Our laboratory has also shown that kb-NB142-70, another novel PKD inhibitor causes dramatic G_2–M cell-cycle arrest in PC3 prostate cancer cells (38). Both of these two PKD inhibitors were also shown to inhibit HeLa cell proliferation (Supplementary Fig. S3). Collectively, our study has identified PKD as a crucial cell-cycle regulator that is activated during G_2–M and promotes mitotic entry. Moreover, in this study, we have provided possible mechanistic insights to PKD inhibitor-mediated cell-cycle arrest, downregulation of cell-cycle regulators and inhibition of cell proliferation, which reflected a positive role of PKD in promoting G_2–M transition by modulating Aurora A protein stability.

Aurora A modulates the function of several cell-cycle regulators critical for G_2–M transition and mitosis, such as PLK1, CDK1/cyclin B, and CDC25C (18) and is essential for key mitotic events, such as centrosome maturation and separation (31), mitotic entry and formation of mitotic spindles (27). Aurora A must be degraded to ensure proper mitotic exit. During late anaphase, it loses its interaction with TPX2 and targeted by anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase for proteasomal degradation (16). Aurora A is also downregulated by Fbxw7, a p53-dependent tumor suppressor (15). Mechanistically, Fbxw7 physically interacts with Aurora A and facilitates the ubiquitination-mediated proteasomal degradation in a Gsk3β-dependent manner. Aberrant expression and proteostasis of Aurora A have been linked to many cancers, thus enabling it as a promising target for therapeutic interventions in cancer (18). Our study has revealed for the first time, a functional link between PKD and Aurora A kinase in G_2–M progression and mitotic entry of the cell cycle. We have shown evidence that PKD is critical for late G_2 cells to enter mitosis, and that abrogation of its activity delays G_2–M

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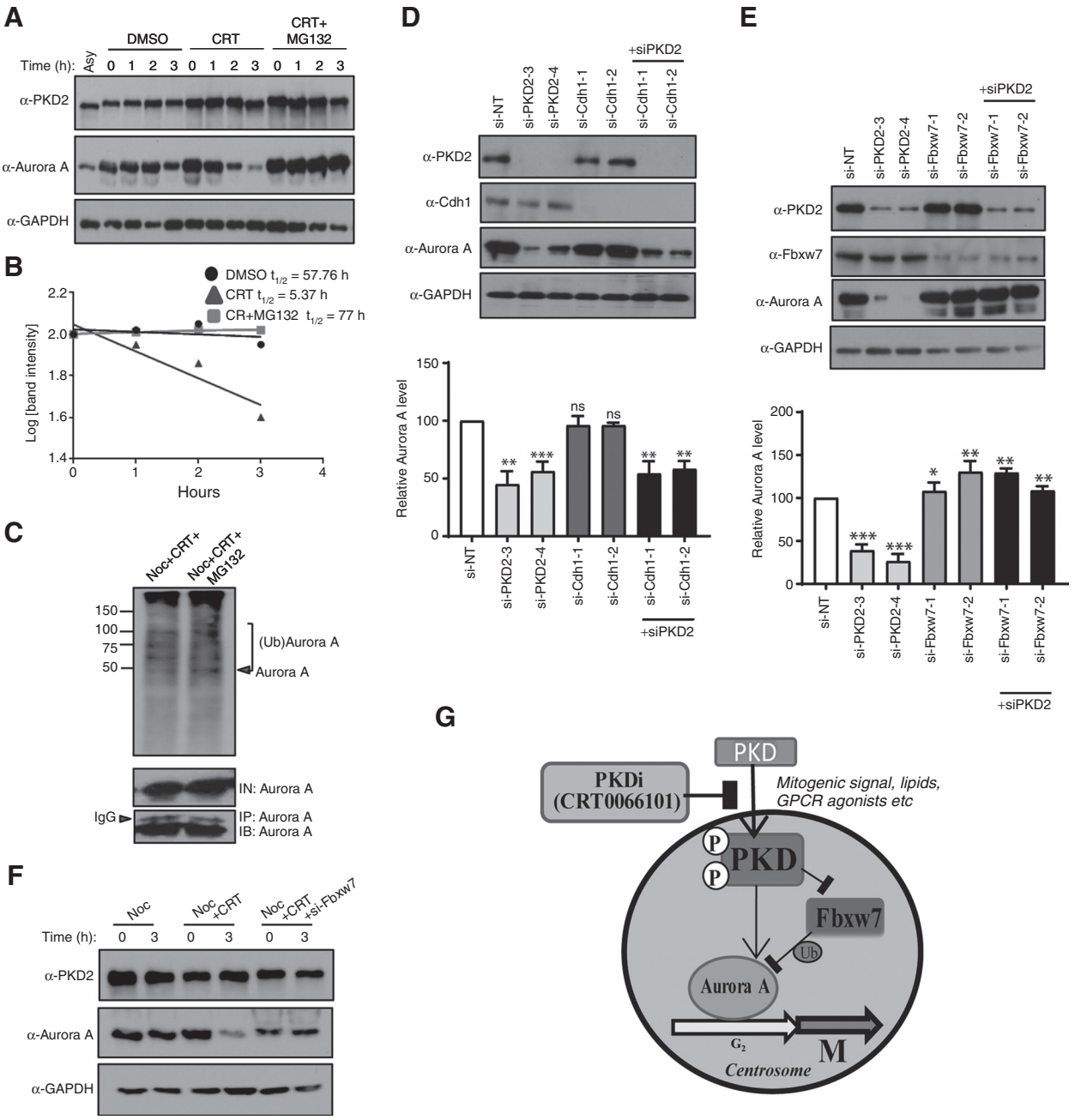

Figure 7.

PKD interferes with Fbxw7-mediated ubiquitination of Aurora kinase A. **A,** Western blot analysis was performed to show that CRT101-mediated downregulation of Aurora A was proteasome-dependent. **B,** Half-life of Aurora A protein was measured from the immunoblot of **A**. **C,** HeLa cells were synchronized at the start of M phase by nocodazole and preincubated with MG132 for 30 minutes. DMSO (control) or CRT101 was added to the culture and the cells were further incubated for 3 hours. Aurora A was pulled down by IP and the protein was analyzed by Western blotting to assess its ubiquitination by CRT101. Downregulation of Aurora A by knockdown of PKD2 and Cdh1 (**D**) or PKD2 and Fbxw7 (**E**) was analyzed by Western blotting (top). Quantification of Aurora A protein levels was performed by densitometry analysis (bottom). **F,** Western blot analysis depicting that CRT101-mediated downregulation of Aurora A is inhibited by knockdown of Fbxw7. GAPDH was used as loading control. **G,** A model of regulation of Aurora A protein stability by PKD. The graph represents average of at least three independent experiments with error bars representing SEM (*, $P < 0.5$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant).

transition (Figs. 2 and 3). We have shown that loss of PKD has profound effects on cell cycle, such as, (i) rapid downregulation of mitotic regulators including cyclin B1, phospho-CDC25^{T48} (Supplementary Fig. S5), (ii) induction of mitotic catastrophe characterized by defects in spindle formation, apoptosis and cell death, (iii) defects in centriole separation in late G_2 and most importantly, (iv) degradation of Aurora A by Fbxw7-mediated proteasomal degradation. Cdh1 is a component of anaphase-

Published OnlineFirst July 17, 2018; DOI: 10.1158/1541-7786.MCR-18-0641

promoting complex that is responsible for substrate degradation during anaphase of mitosis (39). Whereas Fbxw7 (α, β, and γ) expression spans all cell-cycle stages (40), which may be a potential cause for siFbxw7, not siCdh1 to be effective against Aurora A turnover in an asynchronous culture where mitotic cell population is significantly low. Therefore, it is conceivable that a cell harboring inactive or no PKD2 eventually lose Aurora A at the protein level and displays somewhat an "*Aurora A null*" phenotype (41).

Another significant finding of our study is that PKD2 resides on the centrosome during G_2–M and controls centriole separation. We showed that Aurora A and PKD2 colocalized in centrosome in early G_2 and throughout G_2–M. It is known that the maturation and separation of centrosomes are dependent on the recruitment and activation of Aurora A kinase on centrosomes (42). It is unclear whether PKD2 plays a direct role in recruiting Aurora A to the centrosomes. We did not detect direct interaction between PKD2 and Aurora A although both proteins colocalize in the centrosomes and bind to γ-tubulin. Several possibilities may explain this result: (i) the interaction was limited to the centrosomes, which is difficult to detect using whole-cell lysates, (ii) the interaction may be low affinity and transient like many protein kinases and substrates; (iii) the regulation may be indirect through other regulators. Although Fbxw7 was found to mediate the regulation of Aurora A by PKD, it remains to be determined whether PKD directly regulates Fbxw7 activity. Functionally, it is evident that PKD activity modulates the availability/stability of Aurora A at the centrosomes. Thus, inhibition of PKD results in degradation of Aurora A, which may impede the maturation and separation of centrosomes, and thereby block the entry of cells into mitosis. Kienzle and colleagues have shown that PKD positively controls Golgi complex fragmentation at G_2 phase of the cell cycle via Raf–MEK1 pathway, thus, enabling cells to enter mitosis (43). This study is in line with our findings because centrosome duplication and separation is an early step that required for Golgi fragmentation in G_2 (44). Thus, the alteration of Aurora A stability and the resulting defects in centrosome separation may be linked to Golgi fragmentation that occurs at G_2 phase. Beyond Aurora A, the localization of PKD2 in centrosome, the major microtubule-organizing center of the cell, raises the possibility that it might modulate other centrosomal proteins to ensure tight regulation of cell cycle.

In summary, our results provide strong evidence for a novel function of PKD2 in regulating G_2–M transition, mitotic entry and cell-cycle progression. PKD2 does so by protecting critical cell-cycle regulators including Aurora A kinase, an essential mitotic regulator from Fbxw7-mediated proteasomal degradation (Fig. 7G). The regulation of Aurora A by PKD2 provides mechanistic insights to the oncogenic effects of these kinases and may have important therapeutic implications in cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Roy, Q.J. Wang
Development of methodology: A. Roy, M.V. Veroli, Q.J. Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Roy, S. Prasad, Q.J. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Roy, M.V. Veroli, Q.J. Wang
Writing, review, and/or revision of the manuscript: A. Roy, M.V. Veroli, S. Prasad, Q.J. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Q.J. Wang
Study supervision: Q.J. Wang

Acknowledgments

This work was supported in part by the Department of Defense award PC150190 (to Q.W. Wang).

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Received June 14, 2018; revised June 18, 2018; accepted July 10, 2018; published first July 17, 2018.

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Mol Cancer Res Published OnlineFirst July 17, 2018.