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The Role of Protein Kinase D (PKD) Signaling in Breast Cancer
Cell Migration and Invasion

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14. ABSTRACT The goal of my project as outlined in the original application is to analyze the role of Protein Kinase D (PKD) in breast cancer cell motility, the phenotype critical for metastasis. The work I have conducted includes the demonstration that loss of PKD in a number of highly metastatic breast cancer cell lines results in a migration defect. I have also discovered a novel substrate of PKD that mediates growth factor signals to control the cell motility.					
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Introduction

The goal of my project as outlined in the original application is to analyze the role of Protein Kinase D (PKD) isoforms in breast cancer cell motility, the phenotype critical for metastasis. PKD is a serine/threonine kinase that has been best characterized as a key regulator of vesicular fission in golgi trafficking (Yeaman et al., 2004), a process is necessary for the delivery and secretion of molecules destined for the plasma membrane. This highlights its importance in homeostatic cell signaling, but despite four independent studies (Capra et al., 2006; Greenman et al., 2007; Kovalevska et al., 2006; Sjoblom et al., 2006) identifying upregulation and mutations of PKD in cancer tissue, a potential role for PKD in cancer progression has not been well explored. Based in part on a recent study which links PKD to fibroblast cell migration (Prigozhina and Waterman-Storer, 2004), I hypothesized that a PKD signaling network controls cytoskeletal reorganization and cellular adhesion, thereby regulating cell motility. I have tested the migration of PKD1, PKD2, and PKD3-knockdown cells in transwell motility assays using 3T3-conditioned media as chemoattractant. To delineate the specific signaling pathways that control this phenotype, I sought to identify novel effectors of PKD that are necessary in the regulation of motility. I have identified Rabaptin-5 as a novel substrate of PKD that is required for the directed, persistent migration of cells. Additionally, disruption of the PKD – Rabaptin-5 signaling pathway promotes the formation of elongated pseudopodia that drive the rapid invasive migration in 3D microenvironment. These findings provide mechanistic insights into the control of cell motility by a novel PKD – Rabaptin-5 signaling pathway. The results of this research therefore yield an increased understanding of mechanisms that control the metastatic phenotype of breast carcinoma cells, subsequently allowing for new therapeutic strategies targeted to advanced stage tumors.

Tasks According to Statement of Work

Task 1: To determine whether PKD controls cytoskeletal reorganization and a migratory/invasive phenotype in epithelial cells (months 1-15).

- a. Optimize retroviral infection technique to induce over-expression of wild type and various mutant alleles of PKD in various breast carcinoma cell lines (months 1-2).
- b. Optimize lentiviral infection technique to silence expression of PKD in these cell lines (months 3-6).
- c. Analyze the invasive/migratory capacity of PKD-expressing and PKD-silenced cells using a matrigel coated transwell assay (months 7-9).
- d. Characterize actin stress fiber formation and focal adhesion assembly using immuno-fluorescence microscopy and immunohistochemistry (months 10-12).
- e. Analyze the ability of each individual PKD isoform to control invasive migration and influence the dynamics of the actin cytoskeleton (months 13-15).
- f. Characterize breast epithelial cell lines for isoform-specific expression of PKD using RT-PCR (month 15).

Task 2: To investigate PKD isoform-specific mechanisms and substrates that control invasive migration in breast cancer cell lines by conducting migration defect rescue experiments (months 16-28).

- a. Generate mutant alleles of PKD that are refractory to silencing by shRNA and contain mutations in domains of interest and phosphorylation sites in a vector appropriate for retroviral over-expression (months 20-22).
- b. Generate phospho-mimetic and non-phosphorylatable alleles of a panel of known and putative PKD substrates (months 22-24).

- c. Analyze the ability of mutant alleles of both PKD1 and PKD2 that are refractory to silencing by shRNA and contain mutations in domains of interest and phosphorylation sites to rescue migration in PKD-knockdown cells (months 24-26).
- d. Investigate the contribution of a variety of phospho-mimetic and non-phosphorylatable PKD substrates towards invasive migration in PKD1- and PKD2-knockdown cells (months 26-28).

Task 3: To characterize the specific cytoskeletal changes that occur as a result of PKD loss using live cell microscopy (months 29-36).

- a. Conduct time-lapse microscopy to compare efficiency of control and PKD-knockdown breast cancer cells to migrate (months 29-32).
- b. Determine subcellular localization of PKD in breast cancer cell lines using GFP visualization and immunofluorescence (months 32-34).
- c. Analyze specific cytoskeletal differences between control and PKD-knockdown migrating cells using time-lapse microscopy and immunofluorescent staining for cytoskeletal markers (months 34-36).

Body

Task 1: To determine whether PKD controls cytoskeletal reorganization and a migratory/invasive phenotype in epithelial cells

Task 1a – Optimize retroviral infection technique to induce over-expression of wild type and various mutant alleles of PKD in various breast carcinoma cell lines

To fulfill **Task 1a**, we generated mutant alleles of PKD that are refractory to silencing by shRNA and contain mutations in domains of interest and phosphorylation sites in a vector appropriate for retroviral overexpression and optimized this system for expression in breast cancer cell lines.

Task 1b – Optimize lentiviral infection technique to silence expression of PKD in these cell lines

To fulfill **Task 1b**, the ability of two distinct shRNAs to specifically and efficiently reduce levels of PKD1 and PKD2 was tested (**Figure 1**).

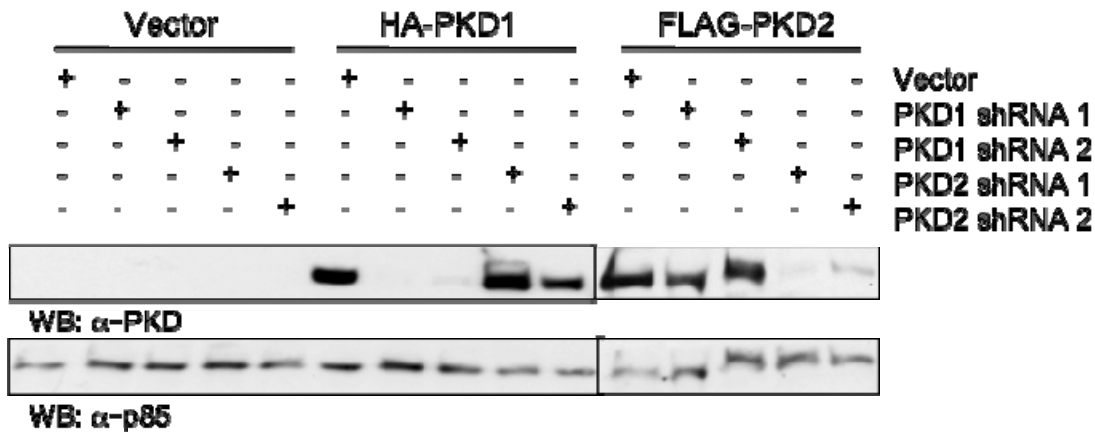


Figure 1: Lentivirally delivered shRNAs specifically and efficiently target PKD isoforms

Task 1c – Analyze the invasive/migratory capacity of PKD-expressing and PKD-silenced cells using a matrigel-coated transwell assay

Overexpression of PKD in cells does not impact cell migration

We employed a gain of function approach to probe the contribution of PKD phosphorylation sites and motifs to the control of cell migration. Highly motile MDA-MB-231 and non-migratory MCF7 cells were co-transfected with HA-PKD WT, HA-PKD S744/748E, HA-PKD K618N, and HA-PKD S916A and S916E mutants and a β -galactosidase construct. After 48 hours, the migration of cells was tested with Transwell migration assays using 3T3-conditioned media as chemo-attractant. Positive transfectants were identified in the presence of the β -galactosidase substrate X-gal, the cleavage of which results in an insoluble blue product. None of the PKD mutants significantly affected the migration of the cell lines tested in the Transwell assay compared to vector-transfected control cells. **(Figure 2)** We discuss our data pertaining to the migratory phenotype of PKD silencing below in **Task 1e**.

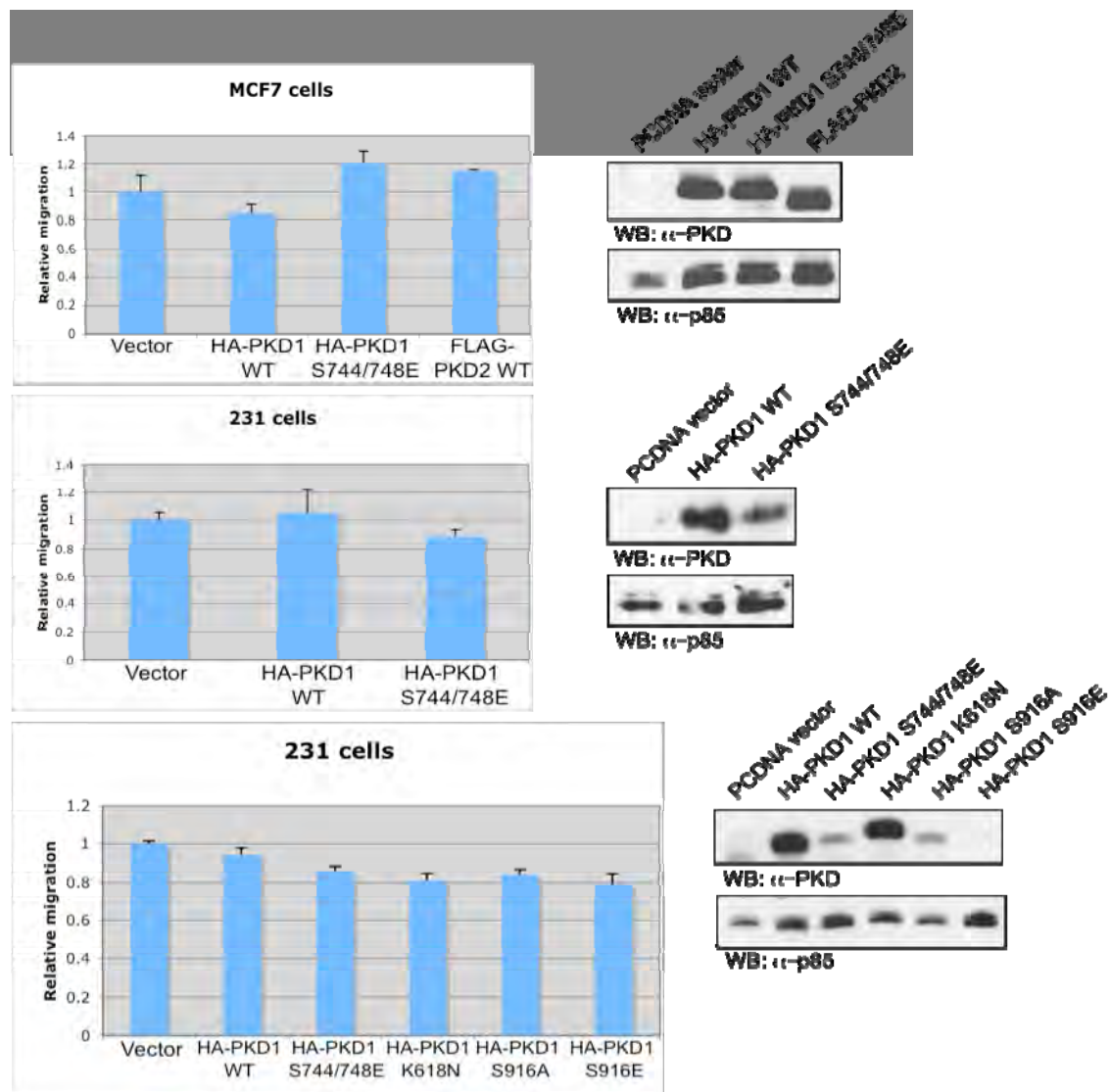


Figure 2: Overexpression of PKD mutant proteins does not impact breast cancer cell migration

MCF7 and MDA-MB-231 cells were co-transfected with PKD mutants or control vector and β -galactosidase (β gal). 48 hours after transfection, cells were subjected to Transwell migration assays using NIH-3T3-conditioned media as chemoattractant. Positive transfectants were identified using the β gal substrate Xgal. Cell lysates were immunoblotted with anti-PKD or anti-p85. Relative migration (y axis) = ratio of the number of migrated cells in test versus control. Error bars represent mean $\pm$ SEM.

Task 1d – Characterize actin stress fiber formation and focal adhesion assembly using immunofluorescence microscopy and immunohistochemistry

Attempts to characterize actin stress fiber formation and focal adhesion assembly in PKD control and knockdown cells were inconclusive. We therefore focused our efforts on PKD signaling mechanisms that control breast cancer cell migration as well as gross morphologic phenotypes controlled by PKD, as discussed below.

Task 1e – Analyze the ability of each individual PKD isoform to control invasive migration and influence the dynamics of the actin cytoskeleton

PKD expression and activation in cancer cells

To evaluate a potential role for PKD in cell migration, we first determined the relative expression pattern of PKD isoforms in a variety of carcinoma cell lines. The aim of this effort was to determine whether there might be a correlation between PKD expression and cancer type in consideration of its metastatic profile. Whole cell lysates were collected from metastatic breast lines (MDA-MB-231, SumPT159, MDA-MB-435, Hs578T), non-metastatic breast lines (ZR75, MCF7), metastatic prostate lines (PC3, Du145), a non-metastatic prostate carcinoma line (LnCap), a metastatic cervix adenocarcinoma line (HeLa), non-metastatic pancreas and colon carcinoma lines (Panc1 and CloneA) as well as non-tumorigenic MCF10A breast epithelial cells and 293T human embryonic fibroblasts. Lysates were immunoblotted with a PKD antibody that recognizes both PKD1 and PKD2 isoforms. There was no obvious correlation between PKD expression level and cancer type. **(Figure 3)**

Cell Line	Origin	Tumorigenicity	PTEN Status	P53 Status	PKD expression
ZR75	Infiltrating ductal carcinoma, ascites	+	+	wild-type	moderate
MCF7	Breast adenocarcinoma, pleural effusion	+	+	wild-type	high
MCF10A	Basal/myoepithelial derived	-	-	wild-type	high
MDA231	Breast adenocarcinoma, pleural effusion	+ (metastatic c)	-	mutant	low
SUMPT139	Breast adenocarcinoma, primary tumor	+ (metastatic c)	-	mutant	high
MDA435	Metastatic ductal adenocarcinoma	+ (metastatic c)	-	mutant	high
HS578T	Ductal carcinoma, primary tumor	+ (metastatic c)	-	mutant	moderate
PC3	Prostate carcinoma	+ (metastatic c)	-	null	high
Lncap	Prostate carcinoma	+	-	wild-type	high
Lncap(A100)	Prostate carcinoma	+	-	wild-type	very high
Du145	Prostate carcinoma	+ (metastatic c)	+	mutant	high
Hela	Cervix adenocarcinoma	+ (metastatic)	+	null	low
293T	Kidney fibroblast	-	+	mutant	very high
Panc1	Pancreas duct, epithelial carcinoma	+	+	mutant	high
CloneA	Colon adenocarcinoma	+	?	?	low

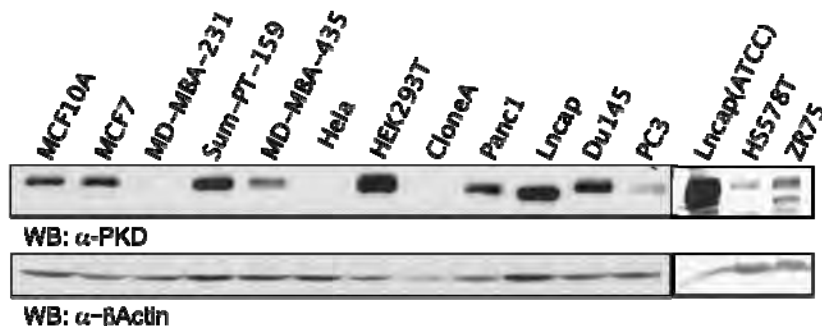


Figure 3: PKD expression across carcinoma cell lines

Whole cell lysates of carcinoma cell lines were immunoblotted using a pan-PKD antibody.

Knockdown of PKD isoforms impairs cell migration

Through its regulation of Golgi trafficking, PKD controls cell motility in NIH-3T3 fibroblast cells (Prigozhina and Waterman-Storer, 2004). To determine a role of PKD isoforms in the invasive migration in breast carcinoma cells, we employed a loss of function approach using the shRNAs we characterized in **Task 1b**. Knockdown of PKD2 but not PKD1 in SumPT159, BT549, and MDA-MB-231 breast cancer cells lines impairs cell migration in a Transwell migration assay using 3T3-conditioned media as chemoattractant (**Figure 4a**). Also, knockdown of PKD3 using two distinct shRNAs separately or in combination did not impair cell migration (**Figure 4b**). These results indicate a dominance of the PKD2 signaling network in the control of this phenotype in these breast cancer cell types. We have therefore discovered an isoform specificity of PKD in the control of breast cancer cell motility.

Figure 4: Knockdown of PKD isoforms impairs cell migration

A) Following Puromycin selection, infected cells were subjected to Transwell migration assays using NIH-3T3-conditioned media as chemoattractant. Relative migration (y axis) = ratio of the number of migrated cells in test versus control. Error bars represent mean $\pm$ SEM. Total cell lysates were subjected to immunoblot analysis.

A

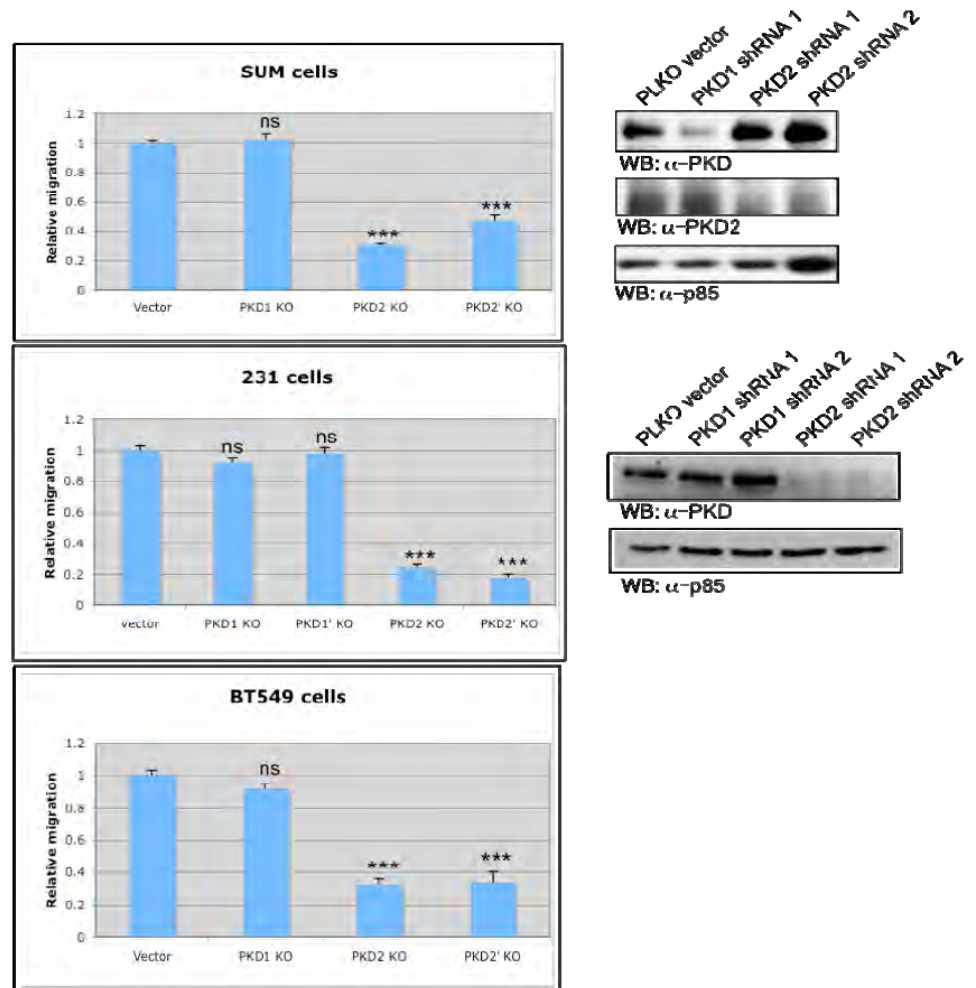
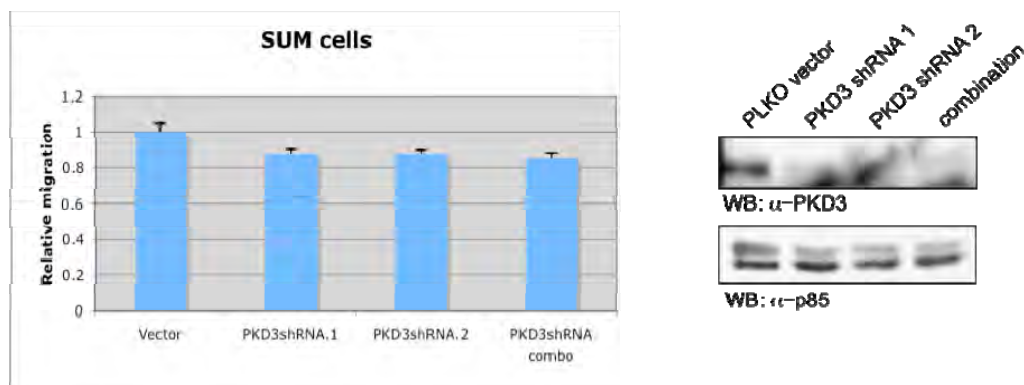


Figure 4 continued

B

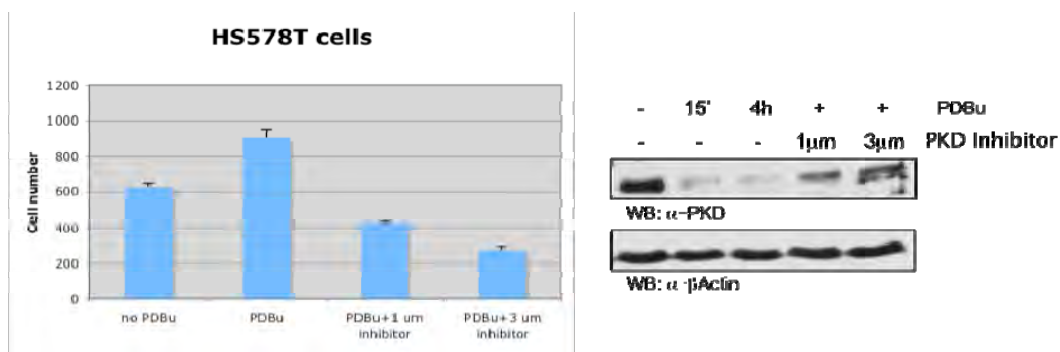


PKD-specific inhibitors impair cell migration

To corroborate our data demonstrating a role for PKD in cell migration, we also adapted a pharmacological approach. Specifically, the role of PKD kinase activity in cell migration was tested using a small molecule, ATP-competitive PKD-specific inhibitor obtained from Cancer Research Technology (Evans et al., 2010). This inhibitor, CRT5, is highly specific for all PKD isoforms and does not target members of the PKC family. Additionally, in a multi-kinase screen, CRT5 at 1 μ M had little effect on the activity of a number of other serine/threonine and tyrosine protein kinases. CRT5 impairs PDBu-stimulated motility of HS578T breast cancer cells in a dose-dependent manner, demonstrating that PKD kinase activity is required for cell migration (**Figure 5**). The migration defect correlated with a loss of PKD phosphorylation, indicating that this defect is due to specific inhibition of PKD.

Figure 5: A PKD inhibitor impairs PDBu-stimulated breast cancer cell migration

Serum-starved HS578T cells were stimulated with PDBu for 15 min in the presence of a PKD inhibitor at the indicated concentrations or DMSO. Transwell migration assays were conducted using 3T3-conditioned media as chemoattractant. Relative migration (y axis) = ratio of the number of migrated cells in test versus control. Error bars represent mean $\pm$ SEM. Total cell lysates were subjected to immunoblot analysis. Note that the PKD antibody has decreased affinity for activated PKD. PKD was analyzed by immunoblotting both 15 min and 4h after stimulation to observe activation before and after the migration assay.



Task 1f – Characterize breast epithelial cell lines for isoform-specific expression of PKD using RT-PCR

To fulfill **Task 1f**, we then determined the relative message expression of PKD isoforms among a subset of these cancer cell lines using real time RT-PCR (**Figure 6**). PKD2 is highly expressed in breast cancer cell lines. PKD1 is minimally expressed in SumPT159 cells but not expressed in the other breast cancer cell lines. PKD1 and PKD2 are differentially expressed in the prostate cancer lines tested and PKD2 expression was negligible in LnCap cells. PKD3 levels are relatively high in all but one cell line tested.

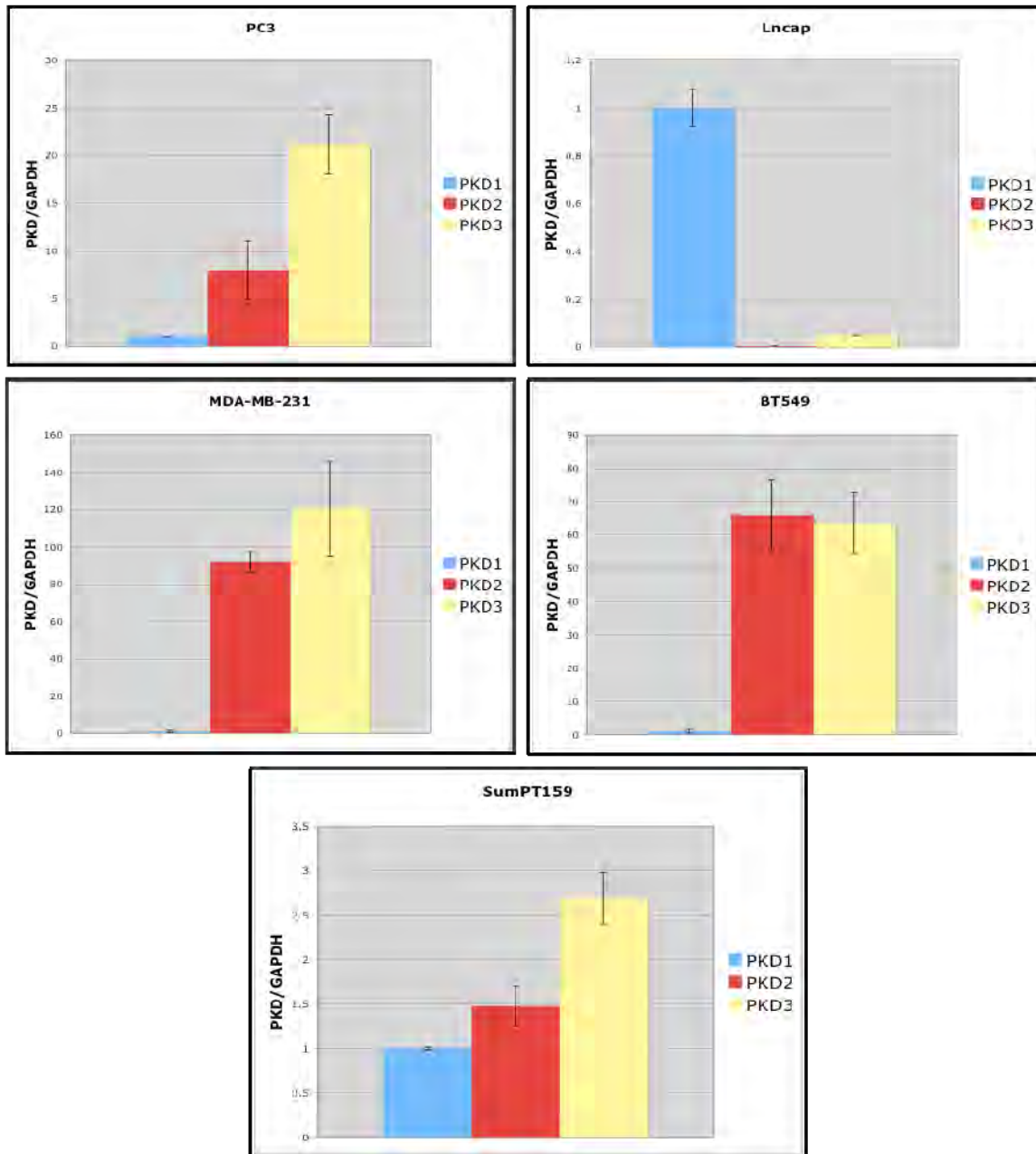


Figure 6: Relative message RNA of PKD isoforms across cancer cell lines

The relative abundance of PKD isoforms was compared in prostate and breast cancer cell lines using real-time RT-PCR.

Task 2 – To investigate PKD isoform-specific mechanisms and substrates that control invasive migration in breast cancer cell lines by conducting migration defect rescue experiments

NOTE – I have described our data pertaining to Task 2c before Task 2b because the rationale for these efforts is most evident in this order.

Task 2a – Generate mutant alleles of PKD that are refractory to silencing by shRNA and contain mutations in domains of interest and phosphorylation sites in a vector appropriate for retroviral over-expression

Overexpression of a non-silenceable PKD allele does not rescue the migration defect

To fulfill **Task 2a**, we generated mutant alleles of PKD that are refractory to silencing by shRNA and contain mutations in domains of interest and phosphorylation sites in a vector appropriate for retroviral overexpression and optimized this system for expression in breast cancer cell lines.

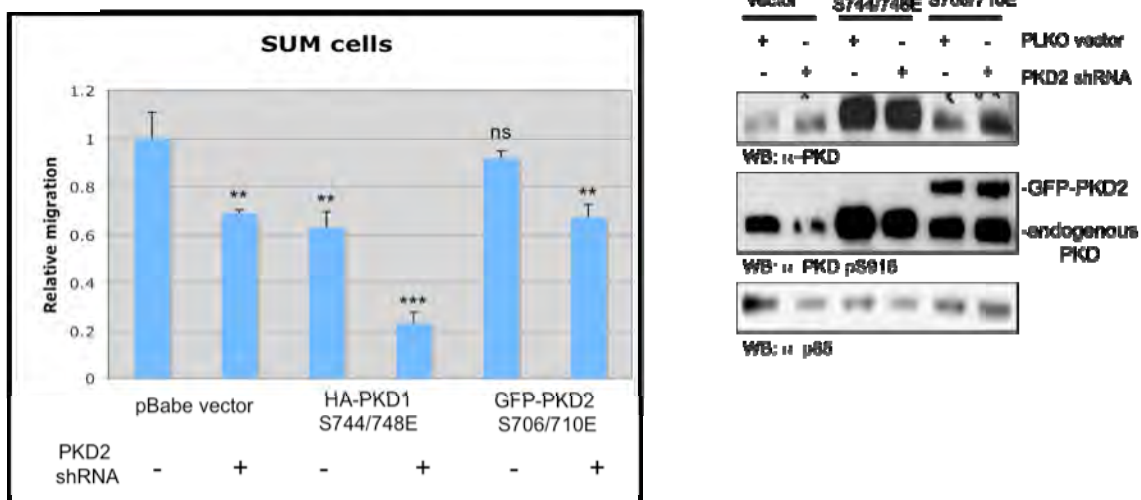
Task 2c – Analyze the ability of mutant alleles of both PKD1 and PKD2 that are refractory to silencing by shRNA and contain mutations in domains of interest and phosphorylation sites to rescue migration in PKD-knockdown cells

To fulfill **Task 2c**, we then determined whether reintroduction of these mutant PKD proteins rescues the migration defect phenotype in PKD-knockdown breast cancer cells. A panel of non-silenceable mutant PKD proteins was used to probe the contribution of PKD phosphorylation sites and motifs in the control of cell migration. To achieve knockdown of both PKD isoforms and expression of the rescue alleles, PKD1- and PKD2-overexpressing breast cancer cell lines were first generated using retroviral infection. After at least two passages in selection media, shRNAs that target PKD2 were delivered lentivirally and cell motility was assayed in a Transwell migration assay. As expected, knockdown of PKD2 impairs cell migration in SumPT159 cells. However, the reintroduction of

constitutively active PKD1 or PKD2 rescue mutants does not restore cell migration in either case (**Figure 7**). Expression of a pBabe-HA-PKD1 S744/748E mutant renders cells less migratory than control and PKD2-expressing cells. To confirm activity of the PKD rescue alleles, lysates were immunoblotted using the PKD pS916 antibody.

Figure 7: Reintroduction of non-silenceable PKD mutants does not rescue the migration defect of PKD2 knockdown in a breast cancer cell line

SumPT159 cells were infected with retroviral empty vector or pBabe-HA-PKD1 WT, pBabe-PKD1 S744/748E or pBabe-GFP-PKD2 S706/710E mutants that are shRNA-resistant. After Puromycin selection, cells were infected with either lentiviral empty vector or PLKO-PKD2 shRNA for 48hr. Cells were subjected to Transwell migration assays using NIH-3T3-conditioned media as chemoattractant. Relative migration (y axis) = ratio of the number of migrated cells in test versus control. Error bars represent mean $\pm$ SEM. Total cell lysates were subjected to immunoblot analysis.



Task 2b – Generate phospho-mimetic and non-phosphorylatable alleles of a panel of known and putative PKD substrates

Rabaptin-5 is a PKD substrate

Because reintroduction of PKD non-silenceable alleles failed to rescue the migration defect of PKD-knockdown cells and over-expression of PKD mutants did not influence breast cancer cell motility, we turned our attention to the identification of PKD signaling effectors that control cell migration to elucidate mechanisms by which PKD controls motility. Since no known PKD substrate had, at the time, been implicated in the control of cell motility, we sought to first characterize a novel substrate that we hypothesized controls cell motility. Rabaptin-5 is an essential effector of Rab-5 in the control of endocytosis. This function is closely linked to the role of PKD in the trafficking of Golgi vesicles. We aimed to determine whether PKD phosphorylation of Rabaptin-5 controls cell motility and whether this signaling influences specific cytoskeletal changes that drive invasive migration.

Phosphoproteomic screens have identified phosphorylation of Rabaptin-5 at Ser407 in a consensus sequence that conforms to the optimal PKD phosphorylation motif (LXRXXS/T) (Dephoure et al., 2008; Villen et al., 2007). The PKD consensus motif surrounding Ser407 is conserved in mammals and other species including fish, flies and worms (**Figure 8A**). A distinct putative PKD consensus phosphorylation motif on Rabaptin-5 is also found at Ser162, although to date no phosphopeptides with this sequence have been mapped by functional proteomics. To determine whether Rabaptin-5 is a PKD substrate, we first transfected LnCap cells with a Myc-tagged Rabaptin-5 construct and stimulated cells with the phorbol ester Phorbol 12,13-Dibutyrate (PDBu) to activate PKD. Immunoprecipitated Rabaptin-5 was immunoblotted with an antibody that recognizes the PKD consensus phosphorylation motif, anti-PKD pMOTIF (Doppler et al., 2005). The reactivity

between Rabaptin-5 and the PKD pMOTIF antibody increases over time upon PKD activation and is attenuated by pre-treatment with Gö6976, a PKC/PKD inhibitor (**Figure 8B**). Phosphorylation of PKD at pS916 serves as a surrogate for PKD activation. In addition, phorbol-12-myristate-13-acetate (PMA), a distinct phorbol ester, as well as PDGF or lysophosphatidic acid (LPA), both physiological ligands of PKD, induce phosphorylation of Rabaptin-5 as detected by immunoblotting with PKD pMOTIF. Interestingly, in response to these stimuli, phosphorylation of Rabaptin-5 coincides with increased total protein levels, suggesting that PKD activation modulates Rabaptin-5 stability (Figure 8B).

Because phorbol esters also activate PKC isoforms and yet other distinct pathways (Griner and Kazanietz, 2007), and because Gö6976 inhibits several other protein kinases (Davies et al., 2000), we next determined the specific contribution of PKD kinase activity to Rabaptin-5 phosphorylation using a molecular genetic approach. HEK293T cells were co-transfected with Myc-Rabaptin-5 and either wild type PKD (PKD WT), a constitutively active PKD mutant (PKD S744/748E), or a kinase inactive mutant (PKD K618N), either in the absence (DMSO) or presence of a proteasome inhibitor (MG132). In control DMSO-treated cells, both WT and activated PKD promote Rabaptin-5 phosphorylation, compared to K617N-expressing cells, demonstrating that PKD activity is sufficient to stimulate Rabaptin-5 phosphorylation (**Figure 8C**). As in Figure 8B, increased Rabaptin-5 phosphorylation coincides with increased total Rabaptin-5 levels. In contrast, in cells pre-treated with MG132, whereas WT and activated PKD stimulate Rabaptin-5 phosphorylation, total levels remain constant under all conditions (Fig. 1C, IP: a-Myc, WB: a-Myc, MG132 panel). Similar results are observed when immunoblotting for phosphorylated and total Rabaptin-5 in total cell lysates. These data indicate that PKD stabilizes and protects Rabaptin-5 from proteasomal degradation. Next, to determine if PKD is exclusively required for phosphorylation of endogenous Rabaptin-5, short hairpin RNA (shRNA)

targeting PKD1 and PKD2 were used. In control cells, PDBu stimulates robust Rabaptin-5 phosphorylation, whereas in PKD1/2 knockdown cells, this is completely abrogated (**Figure 8D**). Taken together, these data show that PKD lies upstream of Rabaptin-5 phosphorylation.

In fulfillment of **Task 2b**, we generated phosphomimetic and non-phosphorylatable mutants of Rabaptin-5. We initially used these mutants to demonstrate a direct phosphorylation event on Serine 407 by PKD and in subsequent experiments, we tested the contribution of these Rabaptin-5 mutants towards invasive migration (**Task 2d**). PKD phosphorylates Rabaptin-5 at Ser407, since expression of activated PKD in cells induces phosphorylation of wild type Rabaptin-5, but not a Ser407Ala mutant (**Figure 9A**). We also observe that in cells expressing activated PKD, there are no significant differences in the total expression levels of Rabaptin-5 Ser407Ala compared to the WT protein. These data indicate that while PKD affects Rabaptin-5 stability through a proteasome-dependent mechanism, it does so in a manner that is independent of Ser407 phosphorylation.

We also show that PKD can directly phosphorylate Rabaptin-5. In an *in vitro* kinase assay, purified PKD directly phosphorylates Rabaptin-5 isolated by immunoprecipitation from cells expressing Myc-Rabaptin-5. Conversely, Rabaptin-5 Ser407Ala is not phosphorylated by PKD (**Figure 9B**). Therefore, PKD directly phosphorylates Rabaptin-5 at Ser407. Since the PKD pMOTIF antibody is not immunoreactive towards Rabaptin-5 Ser407Ala in cells expressing activated PKD (**Figure 9A**), and there is no appreciable phosphorylation of Rabaptin-5 Ser407Ala by PKD in an *in vitro* kinase assay using $\gamma[^{32}\text{P}]\text{-ATP}$, this implies that Ser162 is not phosphorylated by PKD. Moreover, Ser162 phosphorylation has not been detected in the same phosphoproteomic analyses that have identified Ser407 phosphorylation (Dephoure et al., 2008; Villen et al., 2007). While we cannot formally rule out Ser162 phosphorylation, we conclude that PKD exclusively phosphorylates Rabaptin-5 at Ser407.

Task 2d – Investigate the contribution of a variety of phospho-mimetic and non-phosphorylatable PKD substrates towards invasive migration in PKD1- and PKD2-knockdown cells

PKD - Rabaptin-5 signaling regulates the morphology and speed, and persistence of migrating cells (this work was conducted in collaboration with Dr. Jim Norman at the Beatson Cancer Institute in Glasgow, Scotland)

We hypothesize that the PKD - Rabaptin-5 signaling pathway controls cell motility. Since the Rabaptin-5 phosphomimetic and non-phosphorylatable mutants are surrogates for PKD activity/inactivity respectively in this pathway, we tested the impact of expression of these mutants on motility in Rabaptin-5-knockdown cells. Furthermore, because our collaborator, Dr. Jim Norman has developed a system to probe the contribution of PKD in the motility in NIH-3T3 fibroblasts, we demonstrate proof of principle experiments in this line. We are currently testing whether the Rabaptin-5 phosphomimetic mutant rescues migration in PKD-knockdown breast cancer cells.

To determine the contribution of Rabaptin-5 phosphorylation to cell migration, time-lapse images of NIH-3T3 cells expressing Rabaptin-5 mutants subsequent to Rabaptin-5 knockdown were collected. A monolayer was wounded, individual cells migrating into the wound were followed and the persistence and speed of migration were extracted from the track plots (**Figure 10**). Knockdown of Rabaptin-5 or PKD reduces the persistent migration of cells, and this is rescued by expression of siRNA resistant wild type Rabaptin-5 (**Figure 10A and 10C**). In contrast, significantly reduced persistent migration is observed in cells expressing mutant Rabaptin-5 Ser407Ala. We find a reciprocal relationship between the persistence and speed of migrating cells expressing the Rabaptin-5 mutants. While expression of Myc-Rabaptin-5 WT reduces speed, expression of Myc-Rabaptin-5 S407A increases the speed of migrating cells (**Figure 10B**). A requirement for Rabaptin-5 in

directional migration is also demonstrated by an impairment in the overall Forward Migration Index (FMI) of cells expressing Ser407Ala compared to cells expressing wild-type Rabaptin-5 (**Figure 10D**).

Task 3 – To characterize the specific cytoskeletal changes that occur as a result of PKD loss using live cell microscopy.

Task 3a – Conduct time-lapse microscopy to compare the efficiency of control and PKD-knockdown breast cancer cells to migrate.

As noted above, since our collaborator opted to use NIH-3T3 cells as a model system, our initial efforts are not in breast cancer cell lines. We are, however, confirming our findings in breast cancer cell lines. As above, time-lapse images of NIH-3T3 cells expressing Rabaptin-5 mutants subsequent to Rabaptin-5 knockdown were collected. We note a defect in the persistence of the Rabaptin-5 knockdown and Rabaptin-5 S407A-expressing cells to migrate efficiently (**Figure 10**) as well as a morphologic defect described below.

Task 3b – Determine subcellular localization of PKD in breast cancer cell lines using GFP visualization and immunofluorescence.

These efforts are ongoing.

Task 3c – Analyze specific cytoskeletal differences between control and PKD-knockdown migrating cells using time-lapse microscopy and staining for cytoskeletal markers.

We tested whether PKD phosphorylation of Rabaptin-5 acts as a switch between the persistent migration of cells displaying broad lamellipodia and the rapid migration of elongated cells. Rabaptin-5 silencing enhances A2780 cell migration in a 3D Matrigel plug, and this is reversed by expression of siRNA-resistant wild type Rabaptin-5, but not Ser407Ala mutant (**Figure 11A and 11B**) (**This also**

pertains to Task 2d). To determine whether the rapid migration of cells correlates with the pseudopodial extension, the morphology of migrating cells was evaluated. A2780 cells transduced with control non-targeting siRNA (si-nt) displayed broad lamellapodia (**Figure 11C**). In contrast, knockdown of Rabaptin-5 resulted in a slug-like cellular morphology characterized by the extension of elongated pseudopodia. This phenotype was reversed in cells expressing wild-type siRNA resistant Rabaptin-5, but not with Ser407Ala (**Figure 11C**). Pseudopod length was also measured and quantitated (**Figure 11D**). The efficiency of silencing and rescue of Rabaptin-5 mutants is shown in **Figure 11E**. An ongoing effort is the further characterization of these morphologic defects using immunofluorescent staining for cytoskeletal markers. Collectively, these data demonstrate that control of surface receptor trafficking by PKD and Rabaptin-5 signaling modulates cellular morphology to favor either persistent or rapid/random movement. Furthermore, inhibition of this signaling pathway promotes a pseudopodial-driven mode of migration in 3D. All of these findings are currently being confirmed in breast cancer cell lines.

Figure 8A

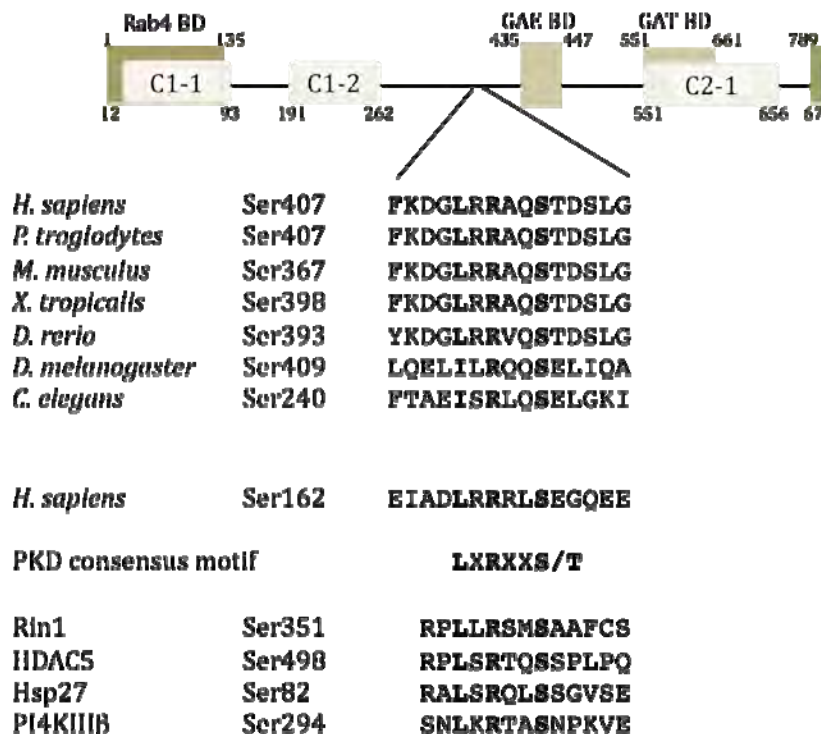


Figure 8: PKD kinase activity is required for recognition of Rabaptin-5 by the PKD pMOTIF antibody

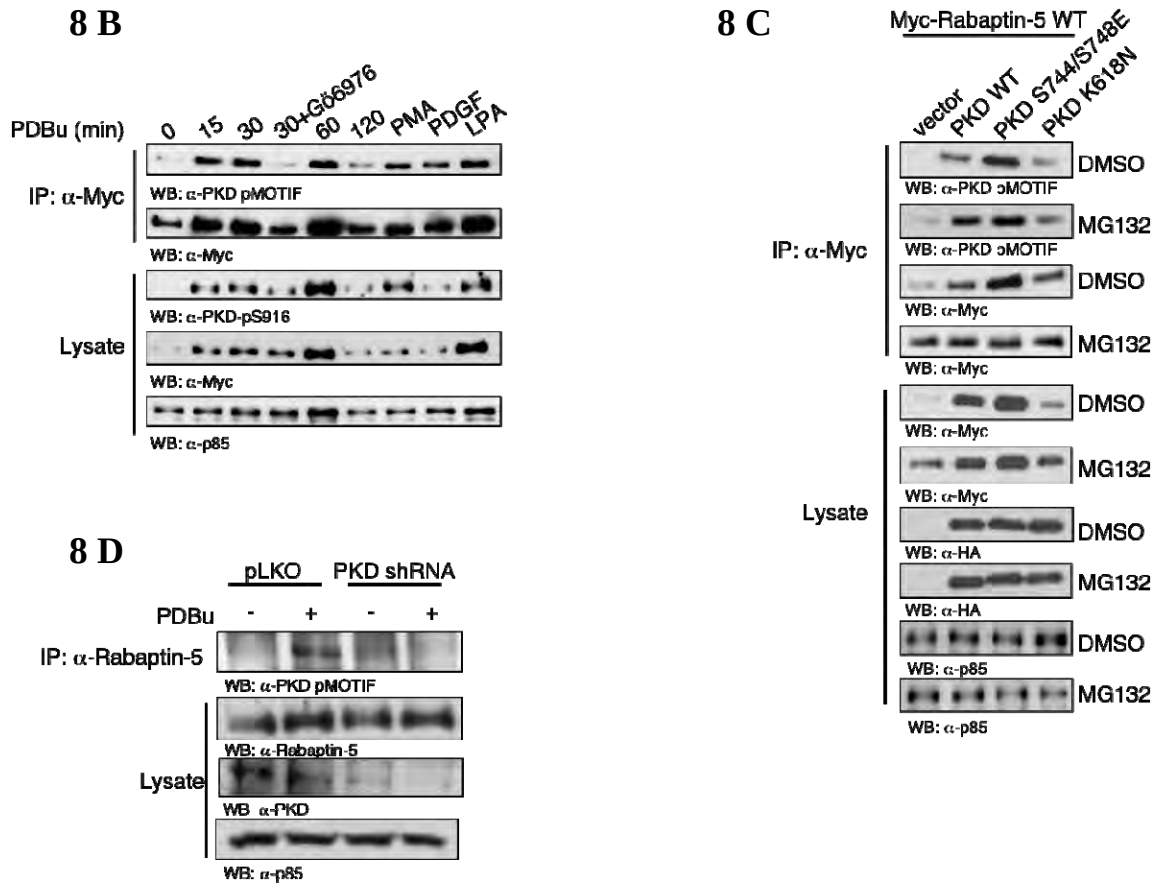
A) Schematic of Rabaptin-5 showing the domain structure and putative PKD consensus phosphorylation site at S407. The S407 PKD motif in Rabaptin-5 is evolutionarily conserved. Rabaptin-5 contains four coiled-coil motifs, an amino-terminal Rab4 binding domain, a carboxy-terminal Rab5 binding domain, and two GGA binding sites (GAE BD and GAT BD). Rabaptin-5 includes a second putative PKD phosphorylation site, S162.

B) PKD-activating reagents promote Rabaptin-5 reactivity with the PKD pMOTIF antibody. LnCap cells were transfected with Myc-Rabaptin-5 and control vector for 48h, then serum starved for 20h. Cells were then stimulated with 1 μ M PdBu, 1 μ M PMA, 10 ng/ml PDGF, 10 μ M LPA, or 10 ng/ml EGF in the presence of the PKD inhibitor, Gö6976, or DMSO over times indicated. Cell extracts were immunoprecipitated with Myc antibody and immunoblotted with the indicated antibodies.

C) PKD kinase activity promotes Rabaptin-5 reactivity with the PKD pMOTIF antibody. HEK293T cells were transfected with Myc-Rabaptin-5 and either HA-PKD WT, constitutively active HA-PKD (S744/748E), or kinase inactive HA-PKD (K618N) and treated with either the protease inhibitor

MG132 or DMSO. Rabaptin-5 was immunoprecipitated from whole-cell lysates followed by immunoblotting.

D) PKD activity is required for phosphorylation of endogenous Rabaptin-5. HeLa cells were lentivirally infected with vector or PKD1 and PKD2 shRNAs in combination. After Puromycin selection, cells were serum-starved and stimulated with 1 μ M PdBu or DMSO for 30 minutes. Rabaptin-5 immunoprecipitates were immunoblotted with the PKD pMOTIF antibody.



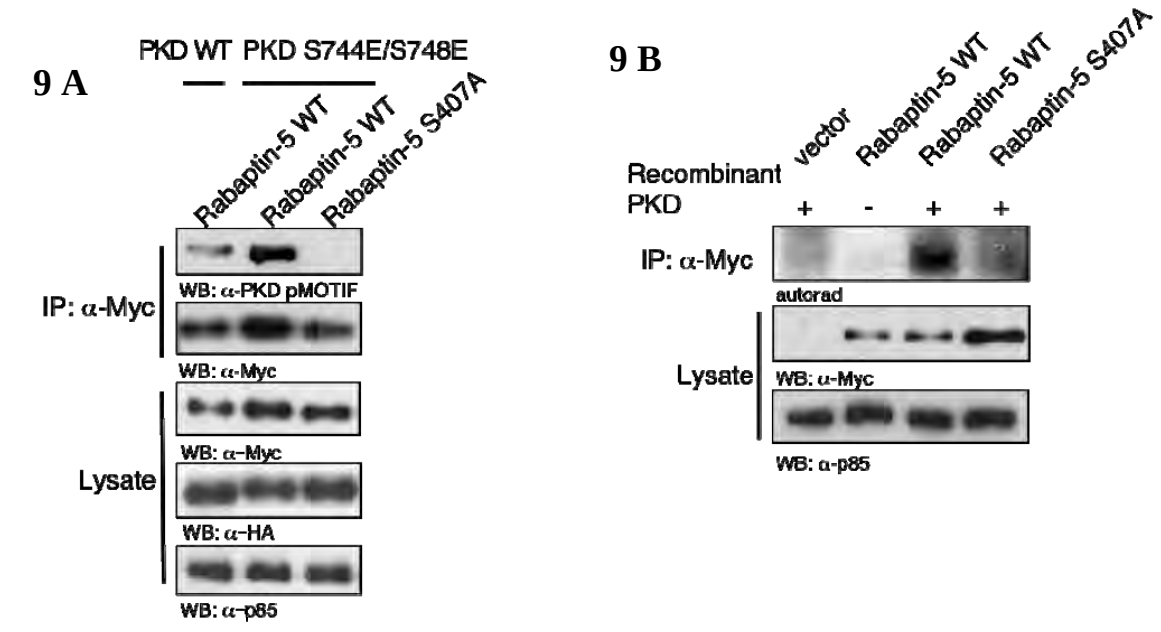


Figure 9: PKD phosphorylates Rabaptin-5 directly at S407

A) PKD phosphorylates Rabaptin-5 at serine 407 in cells. HEK293T cells were transfected with Myc-Rabaptin-5 WT or Myc-Rabaptin-5 S407A and serum starved overnight. Rabaptin-5 immunoprecipitates were probed with the PKD pMOTIF antibody.

B) PKD phosphorylates Rabaptin-5 S407 directly *in vitro*. LnCap cells expressing Myc-Rabaptin-5 WT or Myc-Rabaptin-5 S407A were serum-starved, and Rabaptin-5 was immunoprecipitated with Myc antibody. The immune complexes bound to Protein G beads were incubated in kinase buffer containing both cold and $\gamma^{32}\text{P}$ [ATP] in the absence or presence of recombinant PKD1.

Figure 10

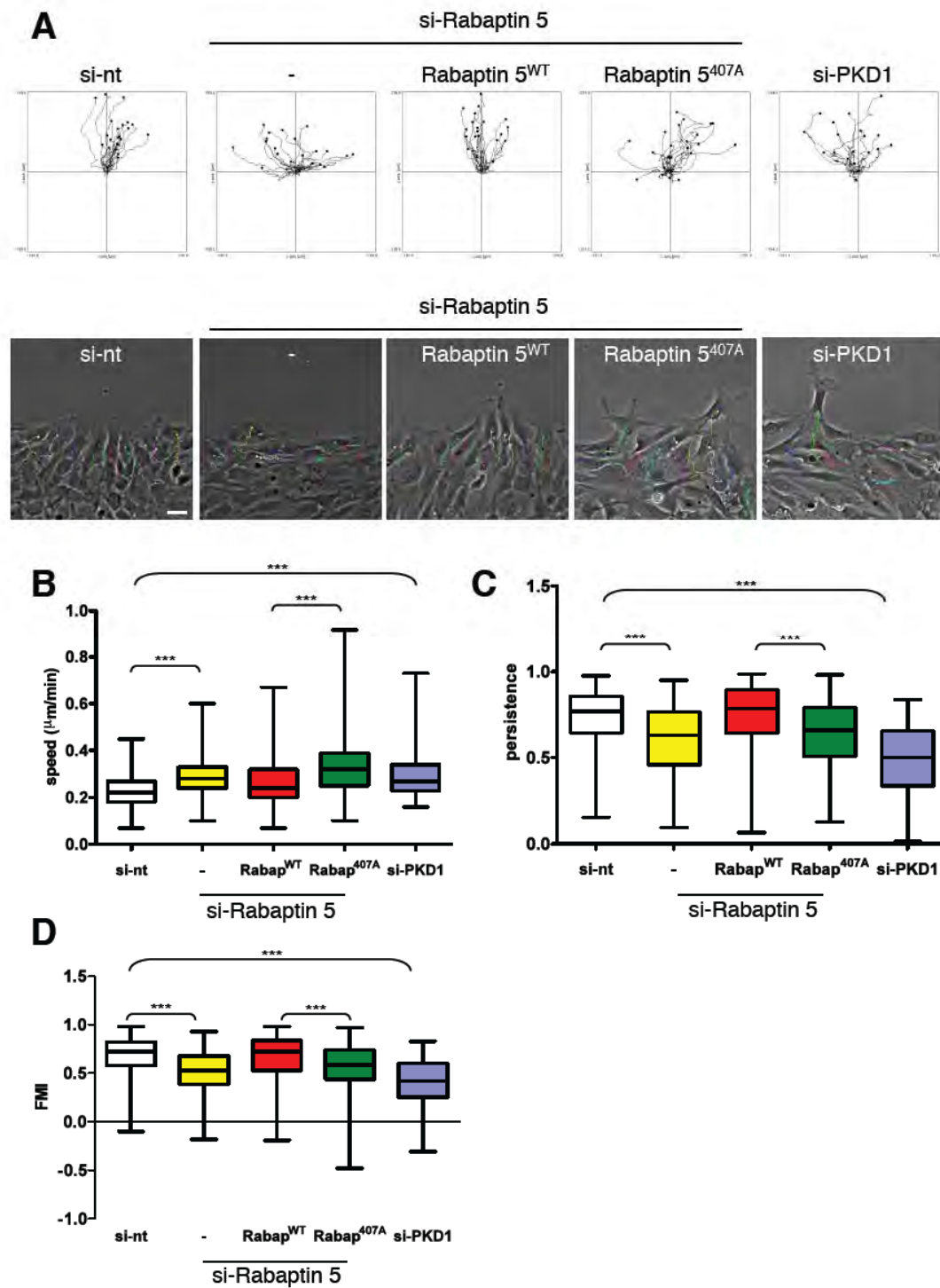


Figure 10: Rabaptin-5 phosphorylation by PKD regulates the persistence and speed of migrating cells

A) Rabaptin-5 phosphorylation is required for persistent migration. Confluent monolayers of cells infected with PKD shRNA or Rabaptin-5 shRNA and expressing non-silenceable Rabaptin-5 mutants were wounded with a plastic pipette tip and the cells were allowed to migrate into the wound. The cells were observed by time-lapse video microscopy, with frames being captured at 20-min intervals. The position of the cell nucleus was followed using cell tracking software, and cumulative track plots of individual cells are displayed in red. Bar, 20 μ m.

B) Rabaptin-5 phosphorylation increases the persistence but decreases the speed of migrating cells. The persistence, speed, and forward migration index (FMI) of migration were extracted from the track plots. Persistence is defined as the ratio of the vectorial distance traveled to the total path length described by the cell. Values are mean $\pm$ SEM; n = 3 independent experiments.

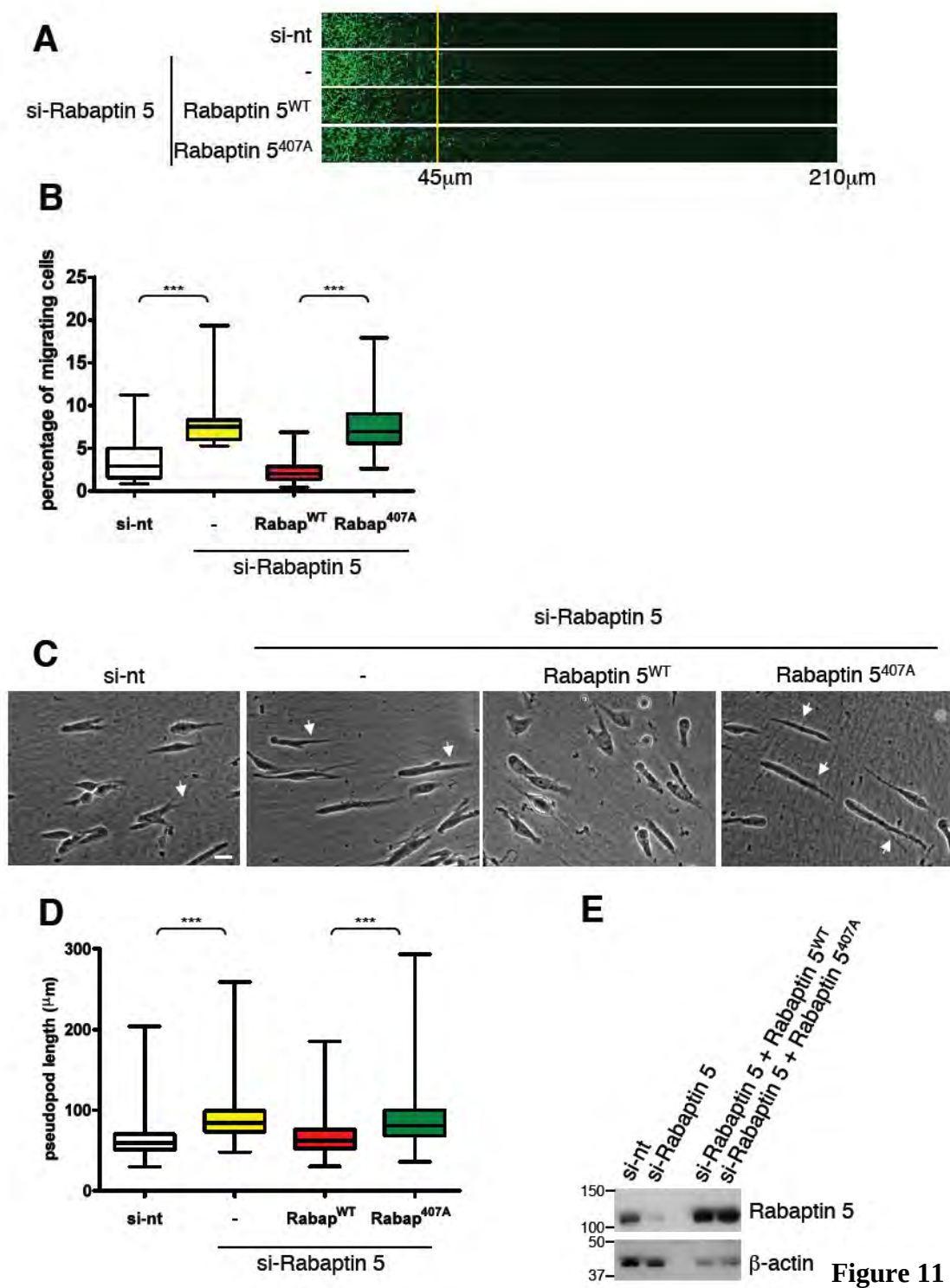


Figure 11: PKD – Rabaptin-5 signaling regulates the morphology and speed of cells migrating in a 3D microenvironment

A) PKD controls the speed of cells migrating in a 3D matrix through Rabaptin-5. Migration of A2780 cells was monitored using an inverted Matrigel plug assay. Invading cells were stained with Calcein AM and visualized by confocal microscopy. Serial optical sections were captured at 15 μ m intervals and are presented as a sequence in which the individual optical sections are placed alongside one another with increasing depth from left to right as indicated. Migration was quantified by measuring the fluorescence intensity of cells penetrating the Matrigel to depths of 45 μ m and greater and expressing this as a percentage of the total fluorescence intensity of all cells within the plug. Data represents mean $\pm$ SEM from three independent experiments.

B) Expression of Rabaptin-5 S407A promotes pseudopod extension. Cells were infected with Rabaptin-5 shRNA followed by transfection with non-silenceable Rabaptin-5 mutants and imaged by confocal microscopy. White arrows indicate cells exhibiting pseudopod extension. Bar, 100 μ m.

Key Research Accomplishments and Reportable Outcomes

- Identified PKD isoforms as critical regulators of breast cancer cell migration
- Demonstrated isoform specificity for PKD2 in the control of breast cancer cell

migration

- Discovered a novel substrate of PKD, Rabaptin-5
- Discovered a key mechanism by which PKD controls migration
- Demonstrated that the PKD – Rabaptin-5 signaling network is required for directed,

persistent migration on a 2D surface

- Demonstrated that disruption of the PKD – Rabaptin-5 pathway promotes the

formation of elongated pseudopodia that drive the rapid invasive migration in 3D

- Developing a manuscript to submit to Developmental Cell

Conclusion

The overarching goal of my project as outlined in the original application has been to analyze the role of Protein Kinase D (PKD) in breast cancer cell motility, the phenotype critical for metastasis. In my first year of study I optimized both overexpression and silencing cell infection systems with which to conduct gain and loss of function experiments using breast cancer cell lines in invasive migration transwell assays. I also worked in collaboration with CRT to develop and test a panel of PKD-specific inhibitors. I was thereby able to determine a reproducible migration-defect phenotype of PKD-knockdown and inhibition in HS578T cells, which are a highly metastatic breast cancer cell line. I also observed a loss on cellular viability caused by PKD depletion which, by means of thorough Propidium Iodide-FACS analysis I found to be an artifact caused by use of a first generation PKD inhibitor. This allows me to conclude that the migration-defect phenotype I observe of PKD-knockdown cells is not due to a general viability issue but instead due to the impairment of the specific signaling networks controlled by PKD.

In the following year of study, I demonstrated that PKD loss by lentiviral shRNA knockdown results in a migration defect phenotype in a number of metastatic breast and prostate cancer cell lines. I further demonstrated that the PKD2 signaling network is dominant in the control of this phenotype in breast cancer cell types Sum159PT and BT549. I confirmed by RT-PCR that both isoforms are present in significant quantities in at least one of these cell types and therefore discovered a potential isoform specificity of PKD in the control of breast cancer cell motility. As a result of this discovery, I decided to broaden the scope of my studies to focus on isoform specific mechanisms and a number of potential substrates that control this phenotype. I therefore created a number of PKD1 and 2 non-silenceable and PKD substrate mutant alleles for PKD-knockdown/rescue analysis.

In my final year of study I sought to gain insights about the signaling and mechanisms of PKD-controlled breast cancer cell migration using these mutant alleles in the transwell migration assay. I developed an efficient PKD knockdown/ over-expression system for use in the transwell migration assays but unexpectedly did not observe rescue of the migration defect caused by PKD knockdown. I therefore sought to identify signaling effectors of PKD in the control of cell migration. I have discovered a novel substrate of PKD that is critical for the directed, persistent migration of cells. Furthermore, disruption of the PKD – Rabaptin-5 signaling pathways results in pseudopodial elongation and rapid random migration. All of these findings are currently being confirmed in breast cancer cell lines.

To summarize, I have demonstrated that the PKD2 isoform is required for cell migration in breast cancer cell lines while, unexpectedly, PKD1, the best-characterized isoform, is not. Furthermore, I have demonstrated that Rabaptin-5, a Rab-5 effector in endocytosis, is a substrate of PKD. This novel signaling pathway is a mechanism by which PKD controls invasive migration.

Summary of Training activities and accomplishments

During the course of my work I have presented posters at meetings cited below, was a Harvard Teaching fellow, and presented my work in a number of seminars. These activities contributed to my training as a pre-doctoral student at the Biological Biomedical Science Program at Harvard Medical School, resulting in the successful defense and completion of my PhD. Much of the work I have conducted under the BCRP fellowship has been submitted and is under review at the high impact journal *Developmental Cell*. I have had the privilege of conducting my studies in a highly collegiate community of top scientists and state of the art facilities. Through a collaboration with Dr. Jim

Norman at the Beatson Institute for cancer research in Scotland, I have discovered a novel signaling pathway that regulates the morphology and invasive migration of tumor cells.

There have been a number of additional mechanisms that have fostered a rigorous and positive training environment. Notably, I have participated in bi-monthly journal clubs in which our lab critically reviews current literature and monthly lab meetings in which each lab member presents his or her work. I have met with my advisor, Dr. Alex Toker on a weekly basis and with my Dissertation advisory committee twice a year. This committee consists of Dr. Sheila Thomas, Dr. Jeffrey Settleman, and Dr. Lewis Cantley, the latter of which is a leading breast cancer research scientist. Dr. Cantley initiated a weekly cancer cell signaling data club, consisting of top labs in the field, in which I have participated and presented my work. Additionally, I have attended a number of seminars in the Harvard Medical School community, including those of Dr. Joan Brugge, who is also a leading breast cancer research scientist. I excelled in the BBS program, in the BCRP pre-doctoral fellowship, and intend to apply my training as a post-doctoral fellow in the field of breast cancer research.

Abstracts

Christoforides C, Toker A. The role of Protein Kinase D in cancer cell motility. Protein Kinases and Protein Phosphorylation FASEB meeting, July 7-12, 2007

Christoforides C, Toker A The role of Protein Kinase D in cancer cell motility. Department of Defense, Breast Cancer Research Program, Era of Hope conference, June 25-28, 2008

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