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Establishing an Ovarian Cancer cell culture model system to study the molecular  
interaction between Src Family Kinases and Protein Kinase A

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Undergraduate Honors Thesis

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**Abstract:** Protein kinase A (PKA) is a cyclic-AMP (cAMP) dependent kinase and is known to regulate many processes, specifically proliferation and migration. PKA activity also plays an important role in the metastasis of ovarian cancer. PKA has been shown to localize to the leading edge of migrating ovarian cancer cells and is required for invasive potential (McKenzie, Campbell et al. 2011). Src family kinases (SFKs) are non-receptor tyrosine kinases that become activated after the stimulation of a variety of plasma membrane receptors. SFKs are proto-oncogenes, that play key roles in signal transduction pathways involved in cell division, motility, adhesion, and survival in both normal and cancer cells. In cancers, SFKs are particularly important in regulating the processes that promote invasion and metastasis. During chronic stress signaling, PKA activates Src through direct phosphorylation (Armaiz-Pena, Allen et al. 2013). The Deming Lab has also shown that active Src family kinases can regulate PKA activity through phosphorylation of the catalytic subunit of PKA at Tyrosine 69. The Deming lab has also made a mutant that cannot be phosphorylated. In the mutant the Tyrosine (Y) has been changed to a Phenylalanine (F) at site 69 (Y69F). Given that phosphorylation at Y69 enhances PKA activity and that PKA and SFKs have been linked in ovarian cancer migration and invasion, I hypothesize that *hypothesize that activation of SFK's induces PKA-C phosphorylation and regulation of downstream PKA signaling*. The goal of my research was to investigate this interaction in wild type PKA catalytic subunit (PKA-C) and Y69F-PKA-C using cell culture and other molecular techniques. The second aim of my project was to design a system utilizing siRNA knockdown technology and rescuing with a C-terminally fluorescently tagged exogenous PKA-C so that later experiments can be aimed at charactering the Y69F-PKA-C.

To address the first aim, I investigated the 1) effect of Src inhibition on global PKA activity in response to epidermal growth factor (EGF) stimulation and 2) the formation lamellipodia response to EGF stimulation when wild type PKA-C is overexpressed or when the Y69 mutant is expressed. The results presented here suggest that 1) Src phosphorylates PKA in response to EGF signaling leading to increased activity and 2) the phosphorylation of Y69 seems to play an important role in the ability of cells to form lamellipodia. The siRNA system proposed shows optimal knockdown of endogenous PKA-C after 72 hours of exposure. Due to the affinity of the siRNA for both endogenous and exogenous PKA-C, silent point mutations were designed and tested to convey resistance to siRNA degradation. These mutations show promising resistance to siRNA degradation and will be used for later experiments to characterize Y69F-PKA-C.

## **I. Background**

### **A. Cell Signaling**

Cell signaling is the complex molecular process by which cells convey messages through integrated protein networks. Extracellular and intracellular proteins mediate the propagation of the signal (Seeger, et al. 1995). Growth factors are polypeptides that stimulate cellular processes by binding to a specific cellular membrane receptor (Goustin, et al. 1986). These molecules are used for short-range cellular signaling as opposed to long range endocrine signaling, and can be found in many different tissues (Goustin, et al. 1986). Growth factors will bind to their receptor and propagate a signal, usually through a kinase phosphorylation cascade. These receptors tend to be receptor tyrosine kinases (RTKs). When the growth factor binds there is a conformational change in the receptor that results in dimerization and subsequently the autophosphorylation of the

cytoplasmic domain of the receptor (Schlessinger 2000). Cell signaling leads to a variety of outcomes, such as cell cycle progression, metabolism, survival, proliferation, differentiation and motility (Schlessinger 2000; Hubbard, et al. 2007). These outcomes are mediated by the downstream kinases that propagate the signal.

## **B. PKA**

PKA, a cyclic-AMP (cAMP) dependent protein kinase, is known to regulate many cellular processes, including proliferation and migration. It is usually activated by a G protein coupled receptor (GPCR) but has been shown to be activated downstream of growth factor RTKs (Caldwell, et al. 2012). PKA is a holoenzyme consisting of two regulatory domains (R) and two catalytic domains (C) (Taylor, et al. 2012). In mammalian cells, both the catalytic and regulatory subunits exist in isoforms (C $\alpha$ , C $\beta$  and C $\gamma$  in human; RI $\alpha$ , RI $\beta$ , RII $\alpha$ , RII $\beta$ ), however it is known that the catalytic subunits are functionally redundant whereas the regulatory subunit isoforms are not functionally redundant. The R subunits have an inhibitor site which binds the active site of the C subunit when they exist as a holoenzyme. RII has a serine residue that RI does not have in the inhibitor site, allowing for a free C subunit to phosphorylate this site, The RI isoforms has either an alanine or glycine, mimicking a PKA substrate and ultimately inhibiting PKA activity (Taylor, et al. 2012). When cAMP levels rise, cAMP can bind to the R subunit. This binding results in a conformational change that releases the catalytic domains (Taylor, et al. 2012). Once the catalytic domain has been released it is free to phosphorylate numerous intracellular downstream targets (Guarino 2010).

Cytoskeletal organization and cellular migration require tightly regulated PKA activity (Howe 2004). PKA activity can either activate or inhibit cytoskeletal regulators. Interestingly, when PKA is hyperactive or inhibited cellular invasion and migration can

be hindered suggesting that PKA activity is spatially and temporally controlled (Howe 2004). An important aspect of PKA mediated cytoskeletal regulation is PKA's localization. A-kinase anchoring proteins (AKAPs) are responsible for subcellular localization of PKA (Diviani, et al. 2001). AKAPs are a family of proteins that bind to the regulatory subunit of PKA and anchor PKA to a specific subcellular location (Jackson, et al. 2002). They consist of two important motifs, the conserved PKA-binding motif and a unique targeting motif. The PKA-binding motif forms an amphipathic helix that interacts with hydrophobic residues at the end of the N-terminus of the R subunit. The unique targeting motif directs the protein complex (PKA-AKAP) to a specific intracellular location (Diviani, et al. 2001). Many AKAPs have been shown to localize to the actin cytoskeleton (Howe 2004). Howe et al. demonstrated the importance of the AKAP-PKA interaction in regards to directed cell migration. They found that when either PKA or AKAP-mediated localization of PKA was inhibited it resulted in the inhibition of directed cell migration in several different types of mammalian cells (Howe, et al. 2005).

PKA anchoring was also shown to be required for the ability of neuronal cells to respond appropriately to axon guidance cues (Deming, et al. 2015). Netrin-1 is a member of the netrin family of axon guidance cues. Netrin-1 signals through the receptor deleted in colorectal cancer (DCC) and can result in cellular functions other than axonal guidance, such as epithelial cell migration. Netrin-1 signaling through its receptor, deleted in colorectal cancer (DCC), was shown to result in PKA activation, phosphorylation of cytoskeletal regulatory proteins and growth cone guidance (Deming, et al. 2015). This signaling was mediated by a PKA-AKAP interaction, namely Ezrin, radixin, and moesin (ERM), which are a family of plasma membrane actin cytoskeleton

cross-linking proteins. Interestingly, there are many known PKA- regulated proteins implicated the DCC/netrin signaling pathway, such as Src (Deming, et al. 2015).

### **C. Src Family Kinases (SFKs)**

SFKs are non-receptor tyrosine kinases that become activated after the stimulation of plasma membrane receptors, including growth factor receptor tyrosine kinases. Src is extremely important in signal transduction pathways that can result in cell division, cellular motility, adhesion and survival (Sen, et al. 2011). Mis-regulation of Src can result in uncontrolled cell growth, and is associated with cancer. SFKs have a conserved organization of their domains. This consists of an N-terminus followed by SH3 (Src homology 3), SH2 (Src homology 2), a linker, kinase domain, and a C- terminus (Parsons, et al. 2004). The SH2 domain is especially important in signaling because it is able to recognize and bind phosphorylated tyrosine (Boggon, et al. 2004). The SH2's availability for binding phosphorylated tyrosine plays an important role in Src regulation. When inactive, the SH2 domain of Src is bound to a phosphorylated tyrosine within the C-terminus. In this conformation, the kinase domain is unable to be phosphorylated. When the C-terminus is dephosphorylated, Src conformation changes to an open domain. This releases the kinase domain allowing Src to be active (Guarino 2010). Tightly controlled regulation of Src is necessary to maintain the balance between proliferation, migration and cell survival.

### **D. PKA and SFKs in ovarian cancer**

PKA has been found to be dysregulated in epithelial ovarian cancer (EOC) lines, many of which are extremely aggressive when they metastasize (Bai, et al. 2006).

McKenzie et al. found that PKA is activated at the leading edge of migrating SKOV3

EOC cells. They also found that when PKA activity is inhibited, cell migration is blocked. Furthermore, PKA activity was dependent and mediated by the anchoring of type-II regulatory PKA subunits (RII). RII is a subunit associated with the holoenzyme (McKenzie, Campbell et al. 2011). When these subunits were inhibited, migration was also inhibited (McKenzie, et al. 2011). McKenzie et al. also showed that the activity of PKA is up-regulated at the leading edge of SKOV-3 cells during invasion. Their data suggests that PKA activity and anchoring are required for invasion and implicate PKA during EOC metastasis.

Many recent studies have shown that chronic stress promotes tumor growth, angiogenesis, and metastasis. One study showed that stress hormones, such as norepinephrine, lead to an increase in the expression of interleukin 6 (IL-6) mRNA and protein levels in ovarian cancer (Nilsson, et al. 2007). Nilsson et al. showed that norepinephrine stimulation activates Src tyrosine kinase, which was necessary for the over-expression of IL-6. These results indicate that stress hormones activate critical signaling pathways in ovarian cancer (Nilsson, et al. 2007). PKA is another protein that is activated through norepinephrine signaling.  $\beta$ -adrenergic signaling is involved in the regulation of many cellular processes, specifically initiation and progression of cancer. The binding of norepinephrine to the receptor promotes metastasis and dissemination of cancer cells when activated by  $\beta$ -adrenergic receptors-mediated activation of PKA signaling pathways (Cole et al. 2012).

PKA is known to activate Src under certain conditions. Importantly, phosphorylation of serine 17 on Src correlated with the aggressiveness and invasiveness of human ovarian cancers (Stork, et al. 2002). An important study demonstrated that



chronic stress signaling through activation of the beta adrenergic receptor enhanced tumor cell migration, invasion and growth by activating PKA, which then activated Src through a phosphorylation event on serine 17 (Armaiz-Pena, et al. 2013). This work suggests that Src is a key regulator in the PKA-mediated signaling network activated by beta-adrenergic signaling. This mechanism appears to enhance tumor cell migration, invasion and growth (Armaiz-Pena, et al. 2013). The phosphorylation of Y69 site of the PKA-C $\beta$  subunit has been shown in a variety of human cancers (*Phosphosite*), specifically ovarian cancer. However, the kinase/s that mediate this phosphorylation and the exact cellular effects are unknown. The Deming laboratory has discovered that SFKs (Src and Fyn) can phosphorylate PKA-C $\alpha$  on tyrosine 69 (Y69). Although this phosphorylation event appears to enhance PKA kinase activity the impact of Y69 phosphorylation on ovarian cancer cell migration remains to be determined.

### **E. Significance**

PKA and SFKs have often been implicated in oncogenesis. (Cho-Chung, et al. 1995). PKA has also been implicated in stress signaling leading to enhanced tumor growth and angiogenesis (Thaker, et al. 2006). Stress signals, such as noreadrenaline, bind  $\beta$ -adrenergic receptors leading to increased cAMP levels and therefore increasing PKA activity (Schöneberg, et al. 1999). It is well known that PKA's holoenzyme isoforms (type 1 and type 2) exist in a strict balance within a cell. Primary human tumors often exhibit a deviation from this balance and *in vitro* experiments have shown that restoring this balance can reverse oncogenesis.

Both PKA and Src have been shown to have altered activity in ovarian cancer (Wiener, et al. 2003; Al-Alem, et al. 2013). Src tends to be overexpressed in late-stage

ovarian tumors and plays an important role in the immortalization of these cells (Wiener, et al. 2003). Furthermore, reduction in Src expression resulted in a decrease in anchorage-independence, vascularization and tumor development in the SKOV-3 late-stage model (Wiener, et al. 2003). Src has also been shown to increase in expression in metastatic ovarian cancer (Wiener, et al. 2003). PKA's signaling pathway is often altered in a variety of cancers (Al-Alem, et al. 2013) and has been shown to play an important role in SKOV3 *in vitro* invasion (McKenzie, et al. 2011). Increased cAMP levels in response to stress signaling has also implicated PKA in increased angiogenesis and malignant proliferation (Thaker, et al. 2006). Furthermore, Armaiz-Pena et al. show a novel mechanism in which stress signals lead to PKA-mediated phosphorylation of Src serine 17 leading to Src autophosphorylating itself on tyrosine 419. These data suggest that this phosphorylation event may be a key molecular switch in downstream signaling and disease progression (Armaiz-Pena, et al. 2013). Given the relationship between SFKs and PKA it is possible that these two not only interact in cancerous models, specifically ovarian cancer, but also possibly intersect in the signaling that ultimately leads to metastasis.

The overall goal of this research was to **investigate the interaction between SFKs and PKA**. Given that phosphorylation of PKA by SFK's enhances PKA activity (unpublished, Deming) and that PKA activity and localization are required for ovarian cancer cell migration and invasion, *I hypothesize that activation of SFK's induces PKA-C phosphorylation and regulation of downstream PKA signaling*. I investigated my hypothesis by looking at the impact of SFK inhibition on EGF-induced PKA activity and tyrosine phosphorylation of the catalytic subunit. Cells expressing WT or non-

phosphorylatable mutant PKA catalytic mutant were assessed for their ability to form lamellapodia or filopodia in response to EGF to determine the functionality of Y69 phosphorylation. My second aim was establish a model tissue culture system that utilizes siRNA to knockdown endogenous PKA and rescue with exogenous wild-type (WT) and mutant Y69F. By establishing this system, we will be able to better characterize the role of SFK phosphorylation of Y69 as well as other PKA non-phosphorylatable mutants.

## **Methods**

***Cell Culture:*** SKOV3 immortalized human ovarian cancer cells obtained from ATCC were cultured in RPMI-1640 containing 10% fetal bovine serum and 2 mM L-glutamine. Cells were passaged once they reach ~80% confluency and were fed with new media in between sub-culturing.

***Aim I: The impact of SFK inhibition on EGF-induced PKA activity and tyrosine phosphorylation of the catalytic subunit***

### ***Measurement of PKA activity via Western blot:***

Cell culture experiments were performed to monitor PKA activity and tyrosine phosphorylation after epidermal growth factor (EGF; 100 ng/mL) stimulation in the presence or absence of selective SFK inhibition using Src-1. Src-1 was used to inhibit SFKs because it is specific to SFKs with few off-target effects. SKOV3 cells were grown to subconfluence (~90%) and then serum starved overnight. The next morning, cells were pretreated with a control (DMSO) or 2mM Src-1 (Sigma) for 30 minutes followed by incubation with EGF for the times indicated. Cells were harvested in lysis buffer and then total protein was determined using the bicinchoninic acid assay (BCA, Pierce).

Equivalent amounts of cell extract was subjected to SDS-PAGE analysis. Protein samples were transferred to a nitrocellulose membrane and blocked in 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS). The membrane was incubated with an antibody that recognizes phosphorylated PKA substrate (phospho-PKA substrate antibody, Cell Signaling Technologies, 1:1000) in order to indicate PKA activity. PKA and tubulin (Cell Signaling Technology; 1: 1000) levels were also blotted for as controls. PKA activity was normalized to tubulin levels using ImageJ densitometry analysis.

### ***Mutagenesis of tyrosine 69 to phenylalanine***

For the alpha catalytic subunit (PKA-C $\alpha$ ), mutant primers were designed using SnapGene software but following primer recommendations in the QuickChange II Site-Directed Mutagenesis protocol (Agilent Technologies). GFP-PKAC $\beta$  plasmids (Origene) were sent to BioBasic to introduce silent mutations in the siRNA target region and to mutate tyrosine 69 to a phenylalanine.

### ***Immunofluorescence and Filopodia, Pseudopodia assessment***

50,000 cells/well were seeded into a 6-well plate containing a coverslip. 24 hours after plating, 3  $\mu$ g mammalian expression plasmids encoding YFP, WT-PKA-C $\alpha$ -YFP or Y69F PKA-C $\alpha$ -YFP were transfected into the cells using polyethylenimine (PEI) at a ratio of 1:5 ( $\mu$ g DNA:  $\mu$ L PEI). Cells were allowed to grow for 24 hours and then serum starved overnight prior to growth factor stimulation. Stimulation was performed the following day, after 48 hours of plasmid expression. Cells were stimulated EGF (100 ng/mL) for 15 minutes, fixed with 4% paraformaldehyde (diluted in phosphate-buffered

saline; PBS) for 10 minutes at room temperature. Fixative was removed and disposed of following appropriate waste removal protocol. Cells were rinsed with 1X PBS and then permeabilized with 0.5% Triton X-100 (diluted in PBS) for 5 minutes at room temperature. Coverslips were washed twice with 1X PBS for 3 minutes each. Coverslips were then blocked in PBS containing 1% bovine serum albumin (BSA) for 15 minutes rocking at room temperature. Coverslips were drained and then placed on 30 $\mu$ L drop containing a 1:50 dilution of phalloidin (Alexa-fluor phalloidin-568, Molecular Probes) in PBS containing 1% BSA. Coverslips were incubated for 20 minutes at room temperature and then washed in 1X PBS 3 times for 3 minutes each and then rinsed with dH<sub>2</sub>O. Coverslips were drained and then mounted onto slides using 25 $\mu$ L of VectaShield Hard Mountant (contains DAPI in excess) as per manufacturer's instructions and viewed under the fluorescent microscope. Cytoskeletal response to growth factor was assessed and imaged microscopically by observing the formation of filopodia and/or pseudopodia. A total of 100 transfected cells was counted and the marked +/- for any projection formation. Formation of these structures was quantified using Image J software. Since the PKA-C alleles were also YFP tagged, localization was also visually confirmed through fluorescent microscopy and quantified.

***Aim II: Establishment of a model tissue culture system utilizing siRNA knockdown of endogenous PKA and rescue with plasmid PKA***

***Plasmids & siRNA***

Two different plasmids were used over the course of establishing this system. The first was a pCDNA 3.1 vector containing either yellow fluorescent protein (YFP) only or

mouse PKA-C $\alpha$  C-terminally tagged with YFP. There is a single endogenous nucleotide difference in the siRNA target region between the exogenous mouse plasmid DNA and the endogenous human sequence. This plasmid was subjected to mutagenesis using QuickChange II Site-Directed Mutagenesis (see below). In later experiments, a pCMV6-AC-GFP vector containing PKA-C $\beta$  C-terminally tagged with turbo green fluorescent protein (tGFP) and its corresponding tGFP antibody (Origene). tGFP is an improved variant of enhanced GFP (eGFP), however other GFP antibodies do not recognize this tag. An empty vector containing only eGFP was used as a control in these experiments.

Table 1 consists of all the siRNA sequences used and the targeted region of the endogenous PKA (Figure 1A and B). This table also shows the mutagenesis primers designed using Snapgene software following primer recommendations in the QuickChange II Site-Directed Mutagenesis protocol (Agilent Technologies) and ordered through Integrated DNA Technologies (IDT). Primers were also designed to introduce the Y69F mutation into both plasmid YFP-PKA-C $\alpha$  and tGFP- PKA-C $\beta$  (figure 2A and B).

### ***PKA-C $\alpha$ and PKA-C $\beta$ knockdown with siRNA***

The siRNAs used are targeted to the human PKA-C $\alpha$  and PKA-C $\beta$  subunits or a scrambled version of the  $\alpha\beta$  siRNA sequence. Due to the functional redundancy of the  $\alpha$  and  $\beta$  isoforms, both were knocked down to ensure that the absence of the  $\beta$  subunit is not being compensated for by the  $\alpha$  subunit, or vice versa. To ensure that any phenotype observed was specific to knocking down  $\alpha$  and  $\beta$  catalytic subunits of PKA, a control scrambled siRNA was used. 100,000 cells/mL were plated per well into a 6-well plate. siRNA (100nM) was transfected using Lipofectamine 24 hours after plating. Cells were

harvested 24, 48 and 72 hours post transfection and whole cell extracts were subjected to SDS-PAGE and Western blot analysis using an antibody directed against the PKA-C $\alpha$  subunit (Santa Cruz Technology) in order to monitor the efficiency of knockdown.

***PKA-C $\alpha$  and PKA-C $\beta$  siRNA knockdown and rescue experiments:***

In order to introduce a silent mutation to render the PKA-C $\alpha$  allele resistant to siRNA targeting, primers were designed using SnapGene as described above. Mutagenesis was performed following the QuickChange II Site-Directed Mutagenesis protocol (Agilent Technologies) using 50 ng of pCDNA3.1 encoding the YFP-PKA-C $\alpha$  fusion gene (see Table 1; Figure 1A). Snapgene was used to design silent point mutations within the siRNA target region (Figure 1B). These mutations were processed through SpliceCenter to ensure that they would be resistant prior to synthesis. Mutant sequences were sent to Biobasic for synthesis. After receiving the mutants, sequence alignment using BLAST was performed to ensure that the correct mutations were introduced (NM\_011100.4). SKOV3 cells were prepared for siRNA knockdown as described above. 18-24 hours after siRNA transfection, 1.5  $\mu$ g mammalian expression plasmids encoding GFP, siRNA-resistant GFP-WT or GFP-Y69F PKA-C $\beta$  were transfected using PEI and then the efficiency of siRNA knockdown and exogenous plasmid uptake was monitored via Western blotting as described previously. The membrane was incubated with a primary antibody that recognizes PKA-C $\beta$  (Santa Cruz Technology, 1:1,000) followed by a secondary horse radish peroxidase (hrp)-conjugated anti-rabbit antibody (1:10,000). After antibody exposure and washing of the membrane, ECL reagents were used to develop the blot. Endogenous PKA-C $\beta$  migrates at ~40 kD, whereas the exogenous tGFP-tagged

PKA-C $\beta$  proteins run at ~60 kD (due to the GFP tag). A tGFP antibody (1:2000; Origene) targeted to the tGFP tag was used to monitor PKA-C $\beta$  expression due to tGFP not being recognized by eGFP antibodies. Tubulin was used as a loading control.

### **III. Results**

#### ***Measurement of PKA activity during growth factor treatment***

PKA and Src are known to be activated downstream of growth factor signaling. Given our unpublished findings that Src can phosphorylate PKA-C on Y69, and that this appears to enhance PKA kinase activity, we investigated whether growth factor-induced PKA activity was dependent upon Src family kinases. To address this, SKOV3 cells were grown to subconfluence, serum starved and then pretreated with the SFK inhibitor Src-1 or a solvent control. Cells were then stimulated with EGF for 30 minutes and the ability of PKA to phosphorylate its substrates was monitored by western blot using an antibody that recognizes phosphorylated PKA substrates. Cells that were not pre-treated with Src-1 showed the expected increase in phosphorylated PKA substrate, thereby indicating an increase in PKA activity, when stimulated with EGF (Figure 3A). Statistical analysis revealed this difference to be significant ( $p = 0.0119$ ; Figure 3B). Cells that were pre-treated with Src-1 did not show a significant increase in phosphorylated PKA substrate ( $p = 0.3434$ ; Figure 3A and B). In fact, Src-1 pre-treated cells that were stimulated with EGF showed significantly less PKA activity than the control cells pre-treated with DMSO ( $p = 0.0109$ ; Figure 3B).

#### ***Filapodia/pseudopodia formation and quantification***

Pseudopodia predominately form at the leading edge of the cell and are a good indicator of cellular migration (Van Haastert and Devreotes 2004). Given that PKA



localizes to the leading edge of pseudopodia (McKenzie, Campbell et al. 2011), both SFKs and PKA play roles in cellular migration (Howe 2004, Sen and Johnson 2011), and that we have shown that Src phosphorylates PKA on Y69, I sought to characterize the cellular phenotype of this phosphorylation event in cells overexpressing wild type (WT) PKA-C $\alpha$  or expressing YFP-Y69F-PKA-C $\alpha$ . Cells were seeded such that would be ~60% confluent the morning of transfection. Cells that were transfected with YFP only served as a control to establish a baseline of endogenous PKA levels. Cells expressing WT-YFP-PKA-C $\alpha$  localized to the actin-rich membrane ruffles and lamellipodia-like structures, formed in migrating cells (Figure 4A), as indicated by the white arrows. In cells transfected with YFP-Y69F-PKA-C $\alpha$  plasmid DNA, the exogenous PKA did not localize to the actin rich filapodia/pseudopodia (Figure 4A), instead it appears to localization the nucleus. All transfections that were treated with DMSO showed minimal lamellipodia-like structure formation. Those that were transfected with WT-YFP-PKA-C did show a significant increase in filapodia/pseudopodia formation in the DMSO treatment compared to the YFP only transfection cells (\*\*p < 0.01), which was probably due to an excess of PKA (Figure 4B). Though the Y69F mutant cells were able to form some filapodia or pseudopodia structures when stimulated with EGF, it was not significantly different compared to the YFP transfected cells that were stimulated with EGF (p = 0.1192), and therefore can be attributed to endogenous PKA activity (Figure 4B). This suggests that Y69 is important in proper PKA localization and PKA-mediated migration. Furthermore, EGF stimulated cells that were transfected with YFP-Y69F-PKA-C showed a significant decrease in filapodia/pseudopodia formation compared to EGF stimulated YFP-PKA-C transfected cells (\*\*\*\*p < 0.0001; Figure 4B).

***PKA-C $\alpha$  and PKA-C $\beta$  knockdown with siRNA***

Though the above experiments provided exciting data, it cannot be used to fully characterize the phenotype of Src phosphorylation at Y69 due to the endogenous levels of PKA. In order to circumvent this problem, a system that utilizes the siRNA knockdown technique and restoration with exogenous PKA will be a useful tool to better characterize this mutant. Cells were seeded such that they would be ~60% confluent when they were transfected with siRNA. Table one shows the sequences that siRNA targets within both subunits of PKAC. Table one also depicts an endogenous difference between human and mouse PKA-C $\alpha$ . The  $\alpha$  and  $\beta$  siRNA sequences are targeted to the mRNA of human PKA-C $\alpha$  and PKA-C $\beta$  and should silence PKA-C protein expression. The scramble should not target the PKA-C ( $\alpha$  and  $\beta$ ) mRNA sequences. Western blotting revealed that PKA-C $\alpha$  was knocked down during the 48-hour and 72 hour time treatments with siRNA but not during the 24-hour time treatment (Figure 5A). Actin was used as a loading control and indicates that there are not equal amounts of protein in each but it is clear that endogenous PKA was knocked down. Densitometry was performed to account for the uneven levels of actin (Figure 5A). When normalized to the corresponding actin levels, there does not seem to be a great difference between the 48 and 72-hour time treatments. For later experimental time tables, the 72-hour exposure to siRNA was chosen and showed nearly complete and consistent knockdown of endogenous PKA-C (Figure 6A).

***Knockdown of endogenous PKA and restoration with plasmid PKA:***

The second key aspect of this system is to be able to restore PKA expression with an exogenous plasmid version that is easily detectable and that contains the Y69F mutation. This allows for the study of the Y69F phenotype when there is only Y69F present in the cell. Based on the knock down results, cells were exposed to the siRNA for

72 hours. Plasmid DNA encoding for mouse PKA-C $\alpha$ -YFP was added 24 hours after the siRNA was added. Cells were harvested 48 hours after the plasmid DNA transfection. Western blots showed that the endogenous PKA was successfully knocked down in cells all treatments (Figure 5B). The exogenous PKA migrates ~60kD. However, Western blot analysis revealed that there was no restoration with exogenous PKA either (Figure 5B). The light exposure shows YFP-PKA only in cells that did not receive any siRNA. The dark exposure shows faint YFP-PKA only in cells that received the scramble siRNA. The experiment was repeated and yielded the same results (Figure 5B). In the dark exposures, there is non-specific banding present. The non-specific banding does not overlap with the suspected PKA bands and can be disregarded. The data presented here suggests that the siRNA is still able to knockdown the exogenous mouse PKA-C $\alpha$ -YFP.

Due to the siRNA's affinity for the PKA-C $\alpha$ -YFP, a silent point mutation was introduced into the siRNA target region (Figure 1A). As stated previously in the methods, there is already a single difference between human and mouse PKA-C $\alpha$  in this region (Table 1). Mutagenesis resulted in successful introduction of the T  $\rightarrow$  C point mutation, which was verified through BLAST alignment (Figure 6A). The sequence was also aligned to human PKA-C $\alpha$  to ensure that the initial A  $\rightarrow$  G between human and mouse sequences was still present (Figure 6B). This mutant was then used in the above described siRNA system. Fluorescent microscopy revealed that cells were expressing the plasmid (Figure 7A). However, when Western blot analysis was performed to confirm endogenous PKA knockdown and exogenous mouse PKA-C $\alpha$ -YFP expression, there was no band at the expected molecular weight under a light exposure. Dark exposure showed a faint band at ~60 kD (Figure 7B). This suggested that either the cells expressing the

PKA-C $\alpha$ -YFP expression were not successfully transfected with siRNA or that there was still partial degradation of the PKA-C $\alpha$ -YFP.

As previously stated, PKA-C $\alpha$  and PKA-C $\beta$  have redundant functions. However, Phosphosite reports that Y69 is preferentially phosphorylated on PKA-C $\beta$ . Due to these reports, primers were designed with additional silent mutations in the hopes of them being resistant to the siRNA within mouse PKA-C $\beta$ . The human and mouse PKAC- $\beta$  are identical in the siRNA target region (Table 1). Therefore, four silent point mutations were introduced into this region by BioBasic (Figure 1B).. Sequence alignment confirmed the presence of all four silent mutations in wild type PKA-C $\beta$  (Figure 8A). Alignment of Y69F- PKA-C $\beta$  also confirmed the presence of these four mutations as well as the mutation of Y69 (figure 8B). The wild type (WT) PKA-C $\beta$  allele was tested in the siRNA system. The PKAC- $\beta$  containing the silent point mutations shows promising resistance to siRNA knockdown (Figure 9). However, antibodies targeted towards PKAC- $\beta$  do not detect the plasmid protein. An antibody against the tGFP tag confirms the expression of the plasmid in the system. The data here suggests that these four silent mutations have made the mouse PKAC- $\beta$  resistant to siRNA, however they are still being tested in vitro to ensure that they are truly siRNA resistant

#### **IV. Discussion**

PKA and SFKs both play important roles in cancer development. Both of these kinase families share similar functions in promoting proliferation, migration and survival (Sen and Johnson 2011, Caldwell, Howe et al. 2012). PKA has been shown to be phosphorylated in the presence of growth factors, such as EGF. Recent work has shown

that when cells were stimulated with platelet derived growth factor (PDGF) PKA was tyrosine phosphorylated, specifically when fibroblasts were undergoing chemotaxis (Caldwell, Howe et al. 2012). The Deming lab has shown that Fyn and Src, not only phosphorylate PKA on Y69 but this also correlates with an increase in PKA activity (unpublished data). Knowing that both of these proteins are often dysregulated in cancer cells (Bai, Feng et al. 2006, Sen and Johnson 2011) and that tyrosine phosphorylated PKA may play a role in growth factor mediated migration, studying the interaction between SFKs and PKA could reveal new information about how these molecules promote the cancer process.

The ovarian cancer cell line SKOV3 are an immortalized human epithelial cell line established from an invasive ovarian tumor that retained their ability to migrate *in vitro* (McKenzie, Campbell et al. 2011) and served as a good cell culture model for studying the interaction between SFKs and PKA. The data here suggests that there is an interaction between SFKs and PKA in response to EGF signaling. As seen in Figure 3A, when Src-1 is used to inhibit SFKs, we see a significant decrease in phosphorylated PKA substrate in response to EGF stimulation. While this may indicate PKA activity, it is not the best method to measure PKA activity. The antibody used, phospho-PKA substrate, recognizes PKA's consensus sequence (RxxS/T) when it has been phosphorylated. Other kinases, such as protein kinase C, have similar consensus sequences that may be recognized by this antibody (Kemp and Pearson 1990). To truly measure PKA's activity, an *in vitro* kinase assay can be performed under the above described conditions to see if Src inhibition impairs PKA activity in response to growth factor signaling.

To better understand between SFK phosphorylation of PKA in response to EGF

stimulation and the role Y69 might play, cells were transfected with either WT-PKA or mutant Y69F-PKA. The data collected suggests that phosphorylation of Y69 on PKA-C is required to correctly localize the catalytic subunit of PKA during cell migration events. Figure 1A, shows that when YFP-PKA-C cells were stimulated with EGF lamellipodia-like structure formation not only increased but showed YFP-PKA-C localization to these structures. In cells transfected with YFP-Y69F-PKA-C alleles, the formation of these structures was impaired and was found to be statistically significant (\*\* $p < 0.01$ ). Statistical analysis also indicated that EGF stimulation of YFP-Y69F-C cells was not statistically different from DMSO treated YFP-Y69F-C cells ( $p = 0.1790$ ). This suggests that cells expressing Y69F are impaired in the formation of pseudopods and/or filopodia. This is further supported by comparing EGF stimulated YFP only cells to both Y69F treatments. The EGF treated YFP only cells served to establish a baseline of endogenous PKA activity. Neither DMSO nor EGF treated Y69F cells was significantly different from EGF treated YFP cells ( $p = 0.0587$  and  $0.1192$  respectively). Since the Y69F-PKA-C $\alpha$  is in excess in these cells, it is likely that Src binds the mutants instead of the endogenous wild-type simply because there is more present in the cell. However, since these mutants cannot be phosphorylated at Y69 in response to EGF signaling, Src is unable to release PKA. This results in impaired pseudopodia formation. There is still endogenous PKA that is being phosphorylated, which contributes to some partial formation of these structures.

However, because these cells still had endogenous active PKA, it is difficult to fully characterize the effect of the Y69F mutant on the formation of these structures. Ongoing experiments are aimed at characterizing migration when only the Y69F PKA-C

mutant is present in the cell. Currently, siRNA-resistant mutants PKA-C (Figure 5) are being tested against our siRNA to validate their resistance. Once this system is fully established, we can clarify the effects of the Y69F mutant on SKOV3 migration and invasion. Furthermore, pseudopod formation is a good way to indicate if a cell can rearrange the cytoskeleton to form migratory structures but more quantitative migration experiments, such as wound-healing assays, donut assays, pseudopod assays and even invasion assays, need to and will be performed once these siRNA-resistant mutants are confirmed. These assays will be used to determine the migratory and invasive potential of SKOV3 cells expressing WT or Y69F PKA-C alleles to determine the impact of this SFK-mediated phosphorylation event. .

The second aim of my project was to establish a cell culture system in which there are no confounding complications due to endogenous levels of PKA. Figure 4A shows that siRNA was successful in knocking down endogenous PKA. The data in figure 5A verifies that PKA was successfully knocked down after 48 and 72 hours of exposure to the siRNA. Densitometry showed that there was not a large difference in endogenous PKA expression between the 48 and 72 hour time treatments (Figure 5B). Later experiments using the knockdown and attempted rescue with plasmid PKA showed that the endogenous PKA was nearly gone at 72 hours of exposure to siRNA (Figure 5B; Figure 7; Figure 9).

Figure 5B shows that the endogenous PKA-C $\alpha$  was successfully knocked down in all treatments. However, figure 5B also suggests that the single nucleotide difference between human and mouse PKA-C $\alpha$  in the siRNA target region (Table 1) is not enough to prevent degradation. In the PKA-C $\alpha$ -YFP transfected cells there is no expression of

PKA-C $\alpha$ -YFP in the cells transfected with  $\alpha\beta$  siRNA. Longer exposure showed that the scramble siRNA treated cells express PKA-C $\alpha$ -YFP. This suggests that the cells were successfully transfected with the plasmid, however they were unable to express PKA-C $\alpha$  due to the exogenous PKA-C $\alpha$  being silenced (Figure 5B). There is no expression of PKA-C $\alpha$ -YFP, and the Tubulin levels are equal among the treatments. Therefore, it can be concluded that the PKA-C $\alpha$ -YFP is not being expressed in cells that also received the  $\alpha\beta$  siRNA.

With the exogenous PKA-C $\alpha$ -YFP being knocked down, it was necessary to design new mutant primers with additional mutations. Mutation primers were designed using SnapGene software and followed primer recommendations in the QuickChange II Site-Directed Mutagenesis protocol and introduced using QuickChange II Site-Directed Mutagenesis kit (Agilent Technologies). Table 1 and Figure 1A depict the mutation that was introduced into PKA-C $\alpha$ . Unfortunately, this mutation was not enough to convey resistance to siRNA degradation (Figure 7B). Though there is partial expression (Figure 7A and B) of exogenous PKA-C $\alpha$ , it is either not in high enough levels, being partially degraded or being expressed in cells that were not successfully transfected with siRNA. Mutation primers were designed using SnapGene software and followed primer recommendations in the QuickChange II Site-Directed Mutagenesis protocol (Agilent Technologies). After receiving the mutants from Biobasic and verifying the presence of these mutations (Figure 8A and B), the same experiments will be repeated using these plasmids to validate their resistance. These plasmids appear to be resistant to siRNA knockdown (Figure 9) and show promise for future applications, such as wound healing, pseudopod assays and the Src inhibition experiments presented here. However, a possible



limitation for future applications is ensuring that cells being examined in a wound healing assay or a pseudopod assay were successfully transfected with both the siRNA and the plasmid PKA. There are two potential methods that come to mind to address this issue. The first is using siRNA that is labeled allowing for transfection efficiency to be assessed. Another method would be establishing a conditional CRISPR-Cas9 cell system in which in the presence of an antibiotic cells no longer express PKA. This would ensure that all cells have no endogenous PKA and would also eliminate the need for siRNA.

The data collected contributes to the public body of knowledge in two ways. The first is that the data discussed here suggests a new SFK and PKA phosphorylation event as well as a possible phenotype. The second is the established model system to study this phosphorylation event. By understanding the interactions between SFKs and PKA, we may be better able to understand, diagnose and treat cancers at the molecular level.

**Acknowledgments:**

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## **References**

1. Al-Alem, L. F., et al. (2013). "Activation of the PKC Pathway Stimulates Ovarian Cancer Cell Proliferation, Migration, and Expression of MMP7 and MMP10." Biology of Reproduction **89**(3): 73, 71-77.
2. Armaiz-Pena, G. N., et al. (2013). "Src activation by  $\alpha$ -adrenoreceptors is a key switch for tumour metastasis." Nat Commun **4**: 1403.
3. Bai, F., et al. (2006). "Analysis of gene expression patterns of ovarian cancer cell lines with different metastatic potentials." Int J Gynecol Cancer **16**(1): 202-209.
4. Boggon, T. J. and M. J. Eck (2004). "Structure and regulation of Src family kinases." Oncogene **23**(48): 7918-7927.
5. Caldwell, G. B., et al. (2012). "Direct modulation of the protein kinase a catalytic subunit a by growth factor receptor tyrosine kinases." Journal of Cellular Biochemistry **113**(1): 39-48.
6. Cho-Chung, Y. S., et al. (1995). "cAMP-dependent protein kinase: role in normal and malignant growth." Critical Reviews in Oncology/Hematology **21**(1-3): 33-61.
7. Cole, S. W. and A. K. Sood (2012). "Molecular Pathways: Beta-Adrenergic Signaling in Cancer." Clinical Cancer Research **18**(5): 1201-1206.
8. Deming, P. B., et al. (2015). "Anchoring of Protein Kinase A by ERM (Ezrin-Radixin-Moesin) Proteins Is Required for Proper Netrin Signaling through DCC (Deleted in Colorectal Cancer)." Journal of Biological Chemistry **290**(9): 5783-5796.
9. Diviani, D. and J. D. Scott (2001). "AKAP signaling complexes at the cytoskeleton." Journal of Cell Science **114**(8): 1431-1437.
10. Goustin, A. S., et al. (1986). "Growth Factors and Cancer." Cancer Research **46**(3): 1015-1029.
11. Guarino, M. (2010). "Src signaling in cancer invasion." Journal of Cellular Physiology **223**(1): 14-26.
12. Howe, A. K. (2004). "Regulation of actin-based cell migration by cAMP/PKA." Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1692**(2,Äì3): 159-174.

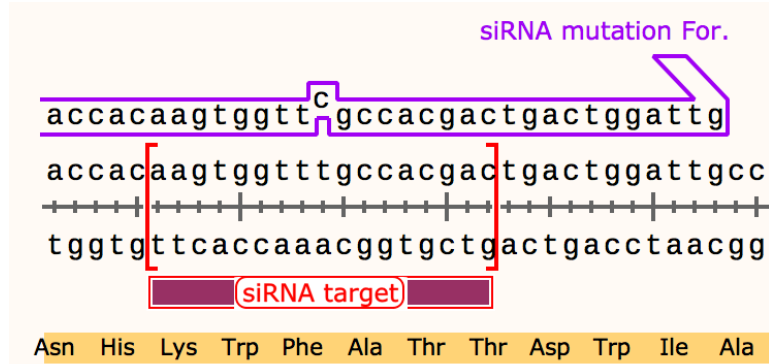
13. Howe, A. K., et al. (2005). "Spatial regulation of the cAMP-dependent protein kinase during chemotactic cell migration." Proceedings of the National Academy of Sciences of the United States of America **102**(40): 14320-14325.
14. Hubbard, S. R. and W. T. Miller (2007). "Receptor tyrosine kinases: mechanisms of activation and signaling." Current Opinion in Cell Biology **19**(2): 117-123.
15. Jackson, S. M. and C. A. Berg (2002). "An A-kinase anchoring protein is required for Protein kinase A regulatory subunit localization and morphology of actin structures during oogenesis in *Drosophila*." Development **129**(19): 4423-4433.
16. Kemp, B. E. and R. B. Pearson (1990). "Protein kinase recognition sequence motifs." Trends in Biochemical Sciences **15**(9): 342-346.
17. McKenzie, A. J., et al. (2011). "Protein Kinase A Activity and Anchoring Are Required for Ovarian Cancer Cell Migration and Invasion." Plos One **6**(10).
18. Nilsson, M. B., et al. (2007). "Stress Hormones Regulate Interleukin-6 Expression by Human Ovarian Carcinoma Cells through a Src-dependent Mechanism." Journal of Biological Chemistry **282**(41): 29919-29926.
19. Parsons, S. J. and J. T. Parsons (2004). "Src family kinases, key regulators of signal transduction." Oncogene **23**(48): 7906-7909.
20. Schlessinger, J. (2000). "Cell Signaling by Receptor Tyrosine Kinases." Cell **103**(2): 211-225.
21. Schöneberg, T., et al. (1999). "Structural basis of G protein-coupled receptor function." Molecular and Cellular Endocrinology **151**(1-2): 181-193.
22. Seger, R. and E. G. Krebs (1995). "The MAPK signaling cascade." The FASEB Journal **9**(9): 726-735.
23. Sen, B. and F. M. Johnson (2011). "Regulation of Src Family Kinases in Human Cancers." Journal of Signal Transduction **2011**: 14.
24. Stork, P. J. S. and J. M. Schmitt (2002). "Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation." Trends in Cell Biology **12**(6): 258-266.
25. Taylor, S. S., et al. (2012). "Assembly of allosteric macromolecular switches: lessons from PKA." Nat Rev Mol Cell Biol **13**(10): 646-658.
26. Thaker, P. H., et al. (2006). "Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma." Nat Med **12**(8): 939-944.

27. Wiener, J. R., et al. (2003). "Activated Src Protein Tyrosine Kinase Is Overexpressed in Late-Stage Human Ovarian Cancers." Gynecologic Oncology **88**(1): 73-79.
28. [SpliceCenter: A suite of web-based bioinformatic applications for evaluating the impact of alternative splicing on RT-PCR, RNAi, microarray, and peptide-based studies.](#) Ryan MC, Zeeberg BR, Caplen NJ, Cleland JA, Kahn AB, Liu H, Weinstein JN. BMC Bioinformatics 2008, **9**:313.
29. *Tyr69 - PKACB (human)*. (n.d.). Retrieved from PhosphoSitePlus database.

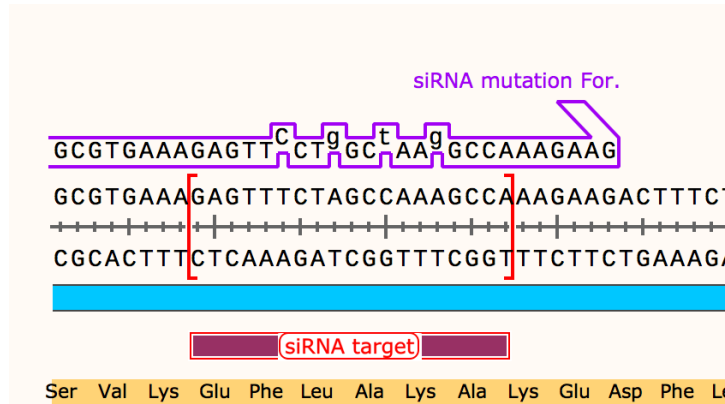
**Table 1:** The table presents the siRNA sequences that were used for the knockdown treatment. There is an endogenous difference between human PKA-C $\alpha$  and mouse PKA-C $\alpha$  indicated in bold. This difference should convey resistance to siRNA knockdown. However, additional mutants were designed in case the siRNA can still target the plasmid mouse PKA-C. Mutations were initially designed in PKA-C $\alpha$  only, but mutations were also designed for PKA-C $\beta$  for later experiments. For the siRNA resistant strands, the differences are also showed in blue.

siRNA	Targeted Sequence		
	Human	Mouse	Amino Acid Sequence
PKA-C $\alpha$	AAG TGG TTT GCG ACA ACT GAC	AAG TGG TTT GCG ACG ACT GAC	LYS-TRP-PHE-ALA- THR- <b>THR</b> -ASP
Proposed siRNA resistant mutations in PKA-C $\alpha$	--	AAG TGG TTC GCC ACG ACT GAC	LYS-TRP-PHE-ALA- THR-THR-ASP
PKA-C $\beta$	GAG TTT CTA GCC AAA GCC	GAG TTT CTA GCC AAA GCC	GLU-PHE-LEU-ALA- LYS-ALA
Proposed siRNA resistant Mutations in PKA-C $\beta$	--	GAG TTC CTG GCT AAG GCC	GLU-PHE-LEU-ALA- LYS-ALA
Control	AAC CGT CGA TTT CAC CCG GGC	--	

Figure 1: Proposed silent point mutations introduced to convey siRNA resistance  
A.



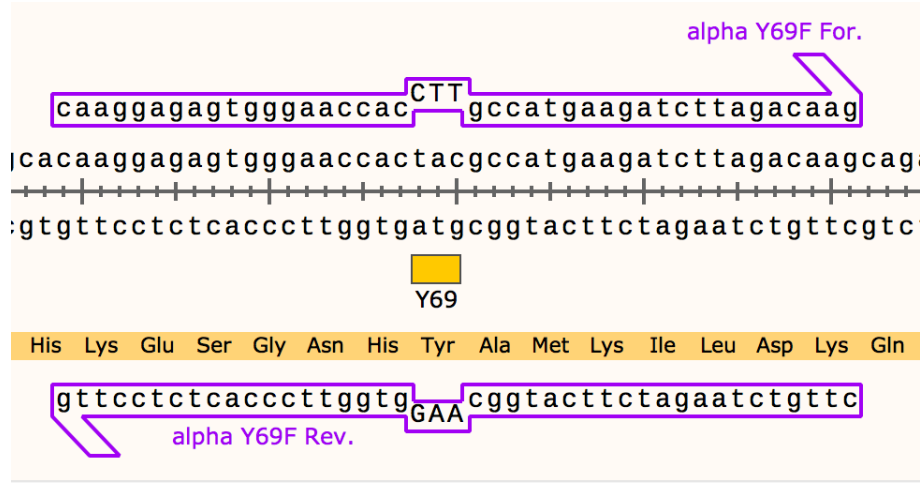
B.



**Figure 1: Silent mutations introduce to produce siRNA resistant plasmid PKA.** Snapgene was used to design silent point mutations in the siRNA target region as indicated. A) A single silent point mutation was introduced into the siRNA target region in mouse PKA-C $\alpha$  (T  $\rightarrow$  C). With this mutation, there are two nucleotide differences between mouse and human PKA-C $\alpha$  in this region. Mutagenesis of this site in PKA-C $\alpha$  was performed using QuickChange II Site-Directed Mutagenesis kit per company protocol. B) Four silent point mutations were introduced into the siRNA target region in mouse PKA-C $\beta$ . Due to this region being identical between human and mouse PKA-C $\beta$  prior to mutagenesis, there is only a total of four silent mutations in the siRNA target region. Mutagenesis these sites in PKA-C $\beta$  was done by Biobasic using the primers designed via Snapgene.

Figure 2: Mutations introduced to create Y69F mutant.

A.



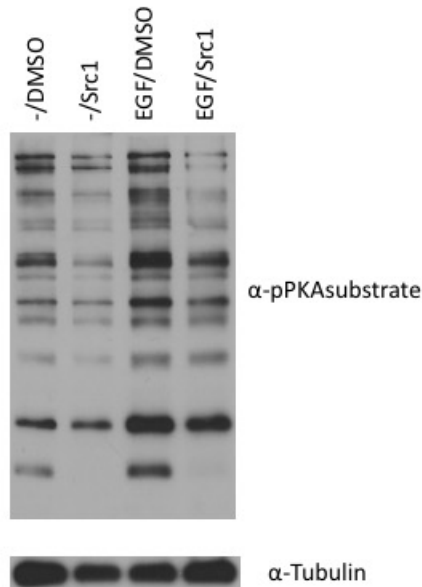
B.



**Figure 2: Tyrosine 69 in both PKAC subunits and the primers used to mutate tyrosine 69 to phenylalanine (Y69F).** Primers were designed using SnapGene following recommendations in the QuickChange II Site-Directed Mutagenesis protocol as stated in the methods. A) Mutagenesis of Y69F in PKA-C $\alpha$  was performed using QuickChange II Site-Directed Mutagenesis kit per company protocol. B) Mutagenesis of Y69F in PKA-C $\beta$  was done by Biobasic using the primers designed via Snapgene.

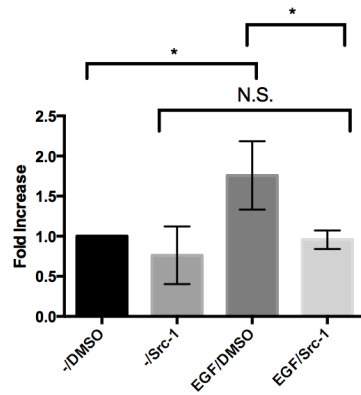


Figure 3: Src inhibition results in decreased phosphorylated PKA substrate during EGF stimulation.



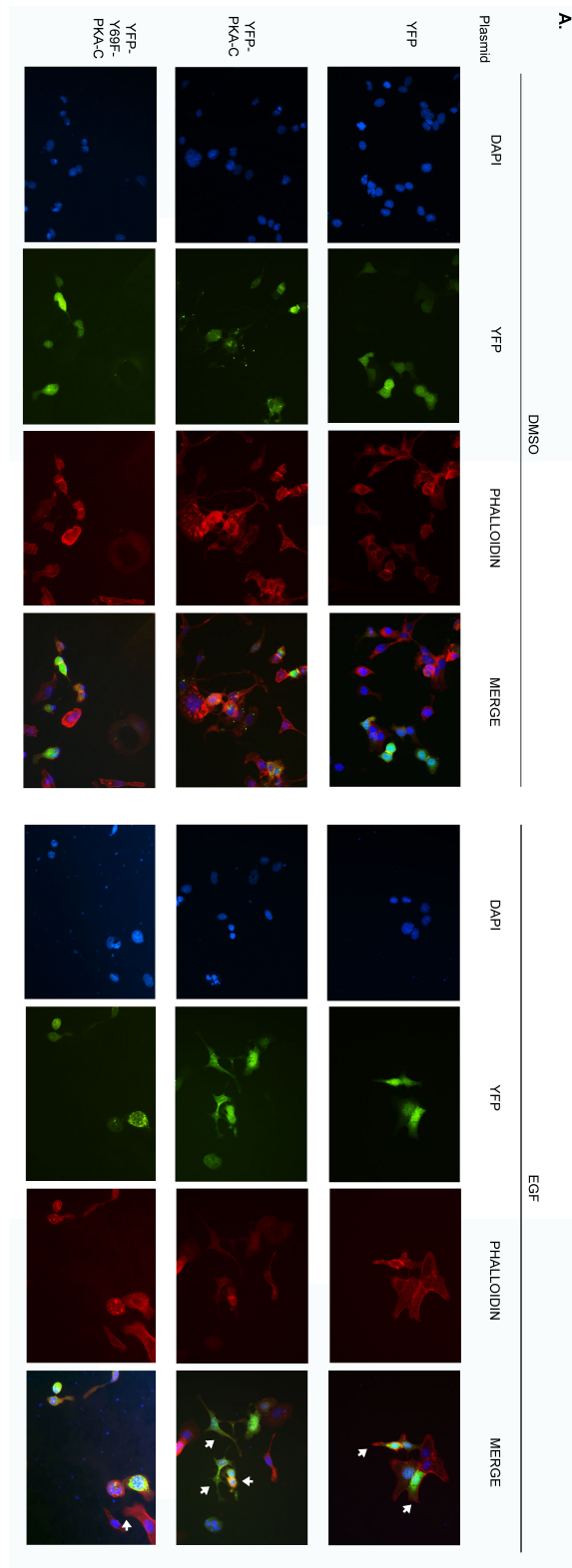
B.

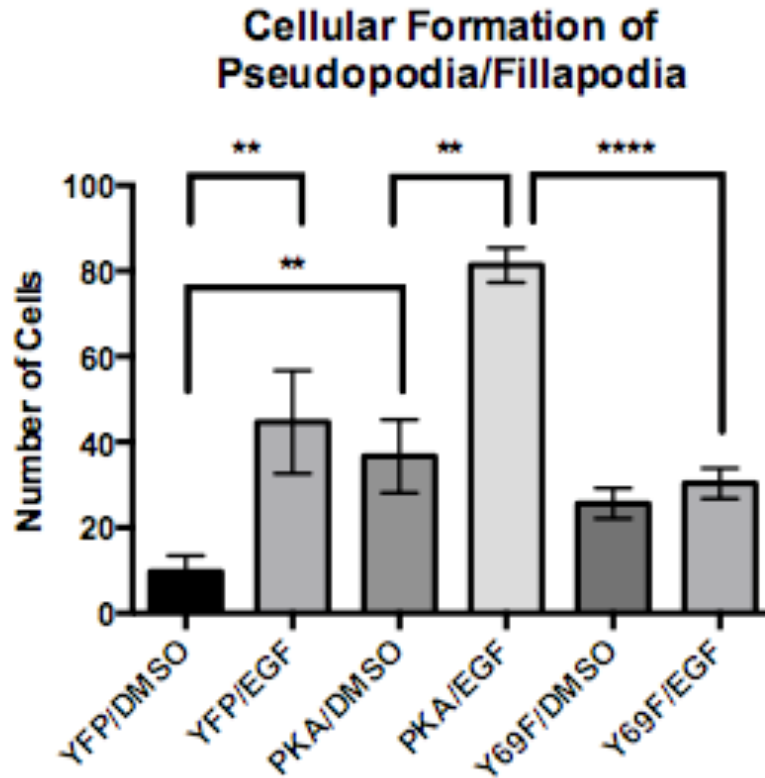
PKA Activity Measured Through Relative Amounts of Phosphorylated PKA Substrate



**Figure 3:** SKOV3 cells were seeded and grown for 48 hours. After 48 hours cells were serum starved for 24 hours. Cells were then pre-treated with either DMSO or 2uM Src-1 (10mM) for 30 minutes. Cells were then stimulated with ddH<sub>2</sub>O or EGF (100 ng/mL) for 30 minutes. **A.** Whole cell extracts were subjected to Western blot analysis of phosphorylated PKA substrate to indicate PKA activity. Tubulin was used as a loading control. **B.** Western blot data was quantified using densitometry via ImageJ. There was a significant difference between DMSO control unstimulated and EGF stimulated ( $p = 0.0119$ ). Phosphorylated PKA substrate was not significantly different when cells were pre-treated with Src-1 ( $p = 0.3434$ ). Src-1 pretreated EGF stimulated cells showed significantly less phosphorylated PKA substrate than the DMSO EGF stimulated control cells. ( $p = 0.0109$ ).

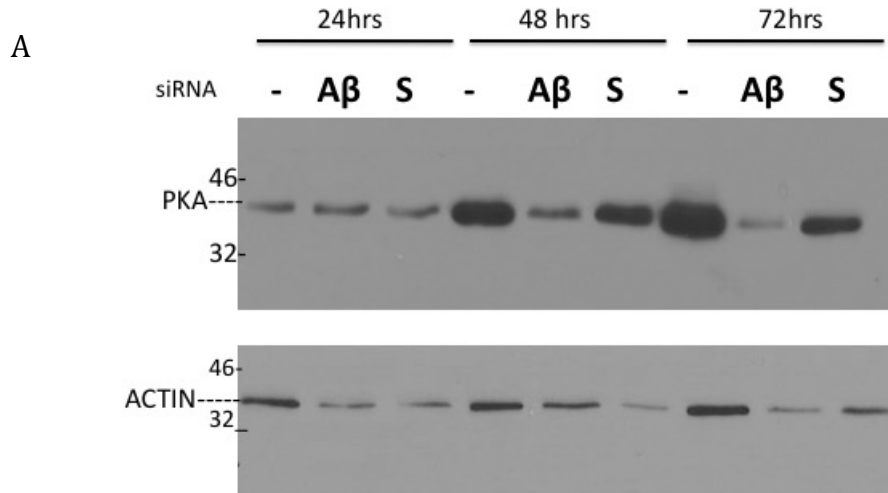
Figure 4: Y69 phosphorylation plays a role in proper PKA localization during lamellipodia formation.



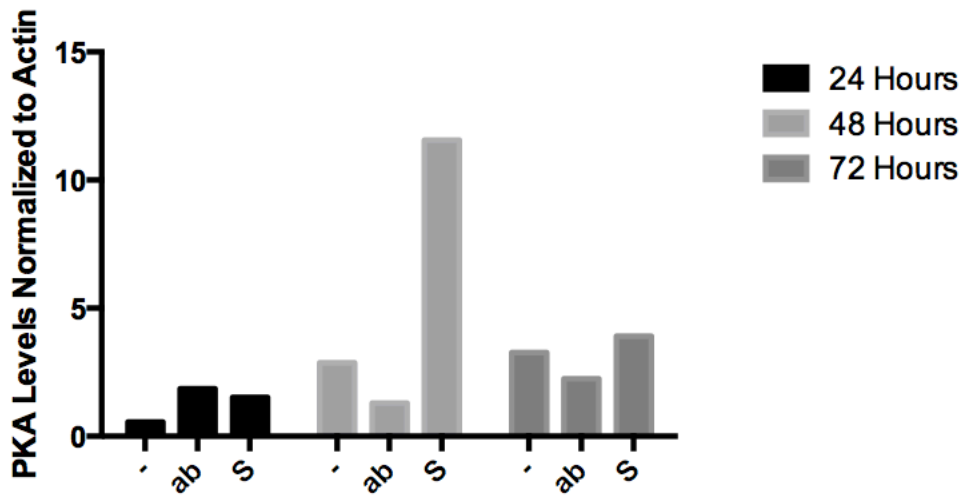
**B**

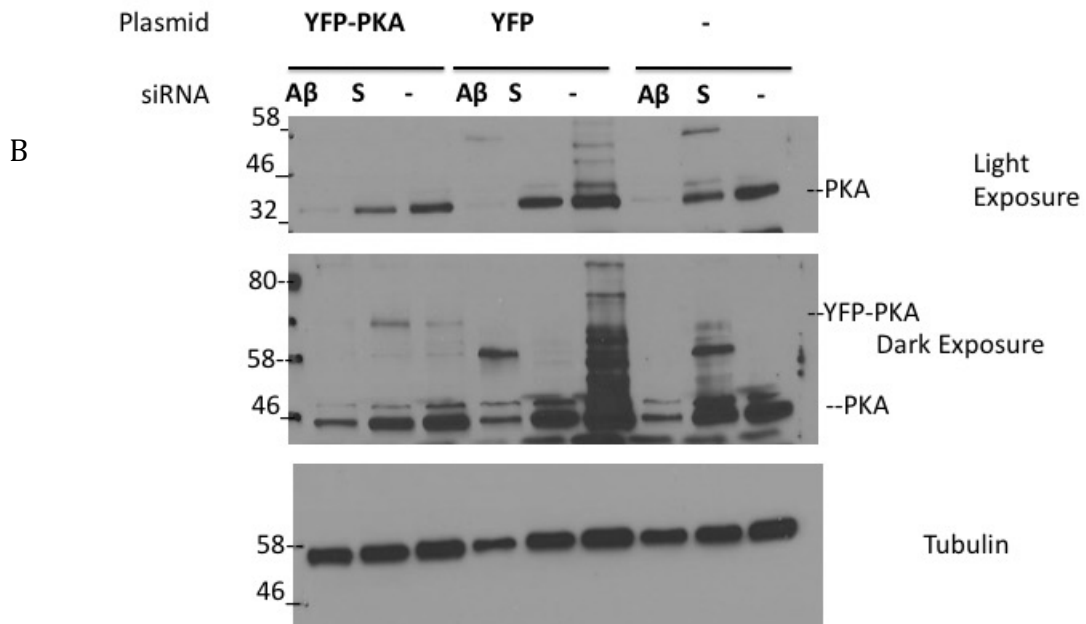
**Figure 4: A.** SKOV3 cells were seeded in a 6-well plate at a density of 50,000 cells/well. After 24 hours of growth, cells were transfected with 3 ug plasmid DNA encoding for either yellow fluorescent protein (YFP), YFP-PKA-C $\alpha$ , or YFP-Y69F-PKA-C $\alpha$  using polyethylenimine (PEI). 24 hours later cells were serum starved overnight. When cells had been expressing plasmid DNA for 48 hours, they were either stimulated with DMSO or epidermal growth factor (EGF, 100ng/mL) for 15 minutes. Cells were then fixed and stained with phalloidin to visualize filapodia and pseudopodia formation and PKA localization and Dapi to denote nuclei. White arrows indicate filapodia/pseudopodia formation in YFP+ cells. **B.** 100 YFP+ cells were counted for each treatment as well as how many transfected cells formed filapodia and pseudopodia formation. Students T-test was used to analyze the data. YFP and YFP-PKA-C $\alpha$  transfected cells showed a significant increase in filapodia/pseudopodia formation when stimulated with EGF (\*\*p < 0.01). YFP-Y69F-PKA-C $\alpha$  transfected did not show a significant change in filapodia/pseudopodia formation when stimulated with EGF (p = 0.1790). Cells that were transfected with YFP-PKA-C and stimulated with DMSO showed a significant increase in filopodia/pseudopodia formation compared to cells transfected with YFP and stimulated with DMSO (\*\*p < 0.01).

Figure 5: Optimization of siRNA knockdown and transfection plasmid PKA containing a single silent point mutation was not enough to convey siRNA resistance.



**PKA levels after siRNA Transfection**

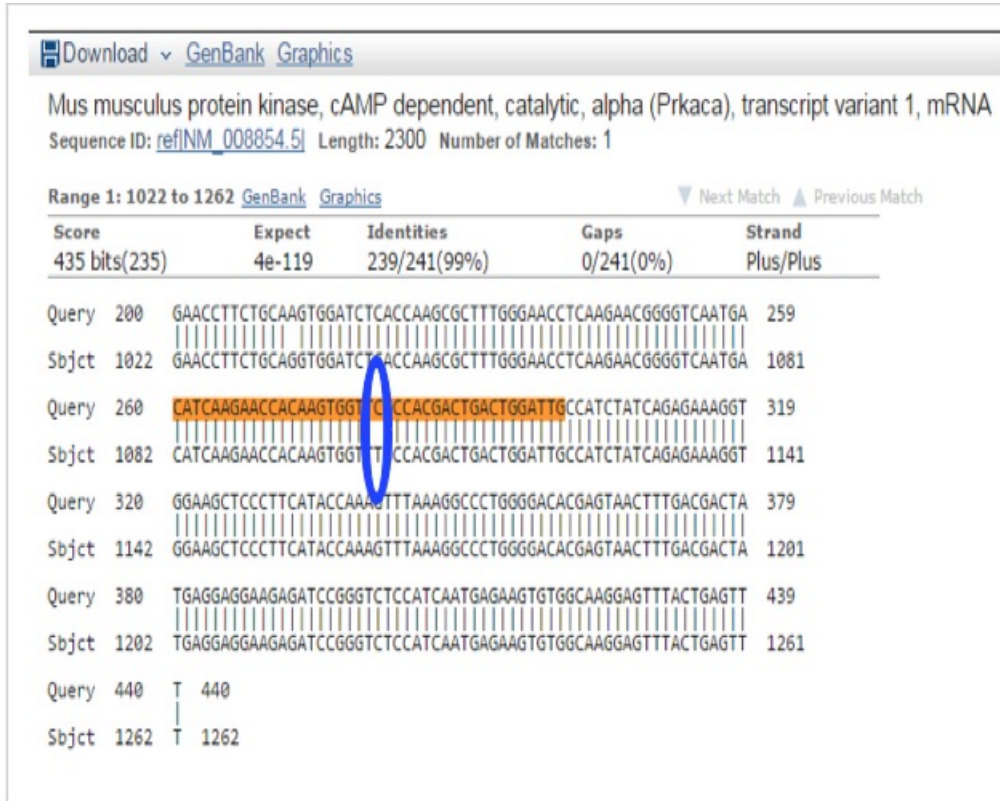




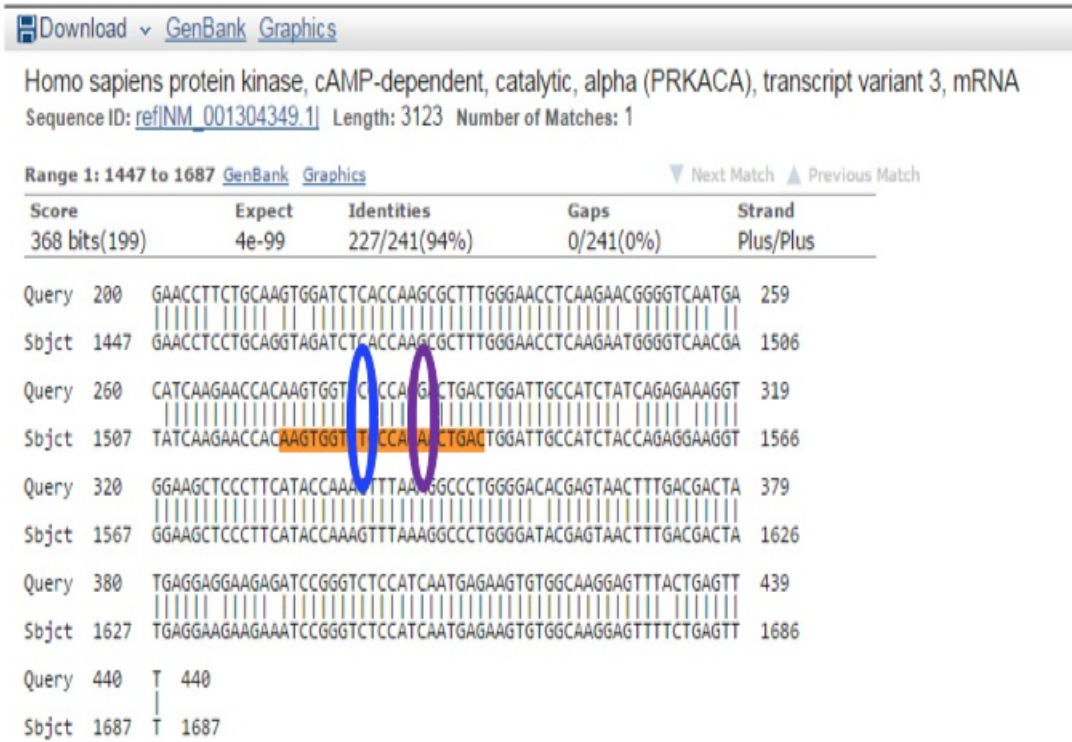
**Figure 3: A: siRNA knockdown optimization.** Cells were plated such that there 75,000 cells per well. Each well received no siRNA, scramble siRNA or  $\alpha\beta$  siRNA. Cells were then allowed to grow for either 24, 48 or 72 hours. Cells were harvested and then a Western blot was performed. 10ug of each sample was loaded into the gel. Actin was used as a loading control. Densitometry was performed, normalizing PKA levels to respective actin levels per treatment. **B:** Cells were plated at 75,000 and 100,000 cells per well. The 100,000 cells per well reached 60% confluency after 24 hours of growth and were treated with siRNA. Cells received no siRNA, scramble siRNA or  $\alpha\beta$  siRNA. Cells were then transfected with YFP plasmid or YFP-PKAC $\alpha$  plasmid 24 hours after siRNA transfection. A group of cells was not transfected with plasmid DNA as a control. The media was changed the following day, allowing for 48 hours of exposure to the siRNA. Cells were harvested 48 hours after the plasmid transfection to allow for enough time to express the plasmid. Light and dark exposures are indicated. Tubulin was used as a loading control.

Figure 6: Sequence alignment to verify the introduction of siRNA silent point mutations into PKA-C $\alpha$ .

A:

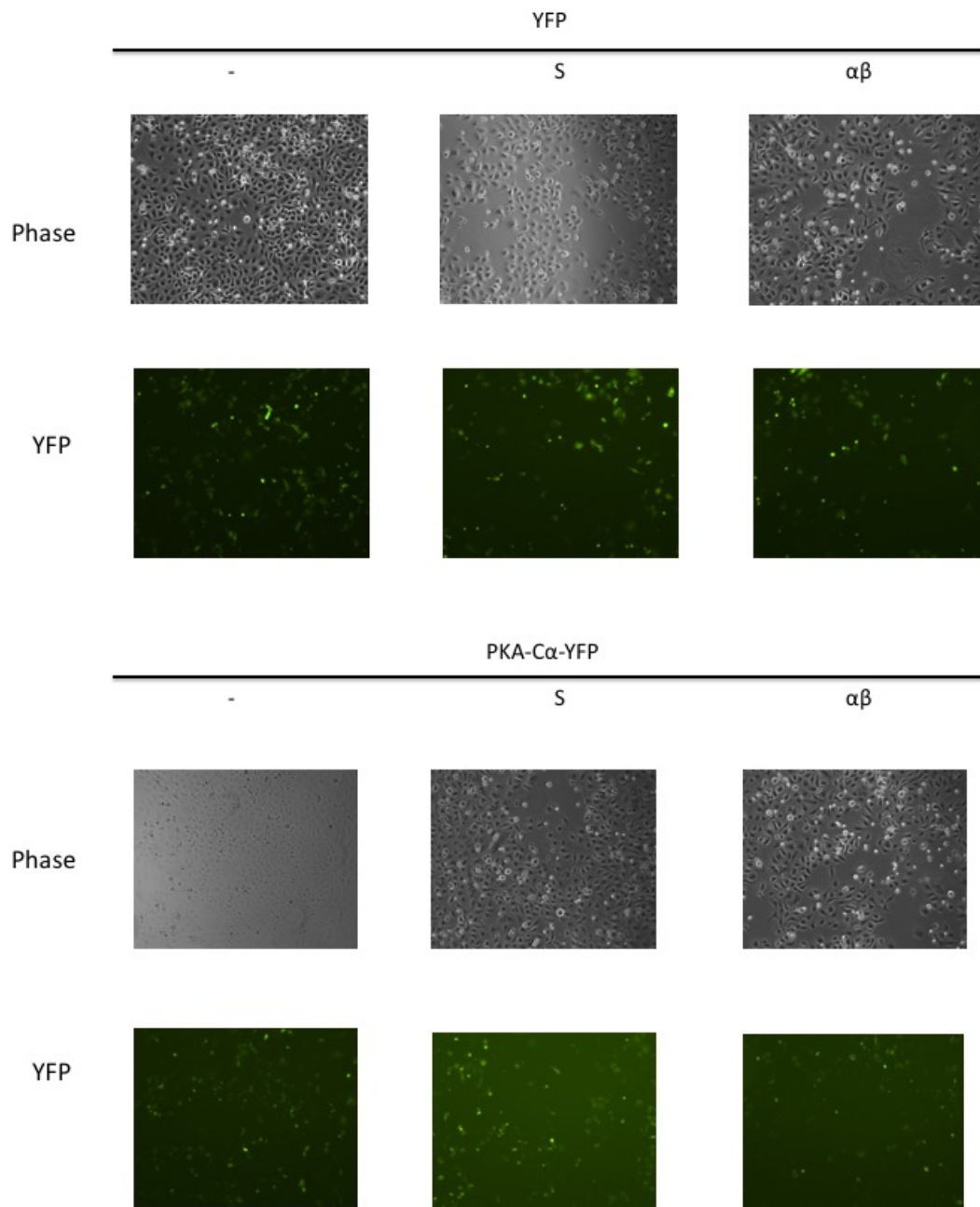


B



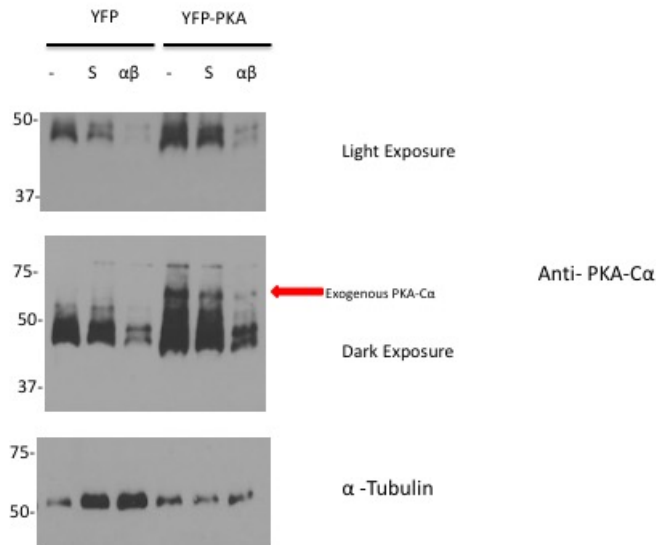
**Figure 4: Sequencing of mouse PKA-C $\alpha$  to ensure the proposed siRNA resistant silent mutation was present after mutagenesis.** Sequencing was done by the Vermont Cancer Center and aligned using BLAST. Lines labeled “Query” are the sample sequences inputted into the database. The “Sbjct” represents the sequence hit within the database that our sample aligned with. A) Alignment of mouse PKA-C $\alpha$  to mouse PKA-C $\alpha$  showed that the T  $\rightarrow$  C mutation was introduced (indicated by the blue circle). Highlighted in orange is the siRNA target region in mouse PKA-C $\alpha$ . B) The mouse PKA-C $\alpha$  sequence was also aligned to human PKA-C $\alpha$ . This alignment shows the T  $\rightarrow$  C mutation (blue circle) as well as the endogenous nucleotide difference between human and mouse PKA-C $\alpha$  at this region (A  $\rightarrow$  G; Purple circle). Highlighted in orange is the siRNA target region in human PKA-C $\alpha$ .

Figure 7: Restoration with two silent point mutations did not convey siRNA resistance.  
A





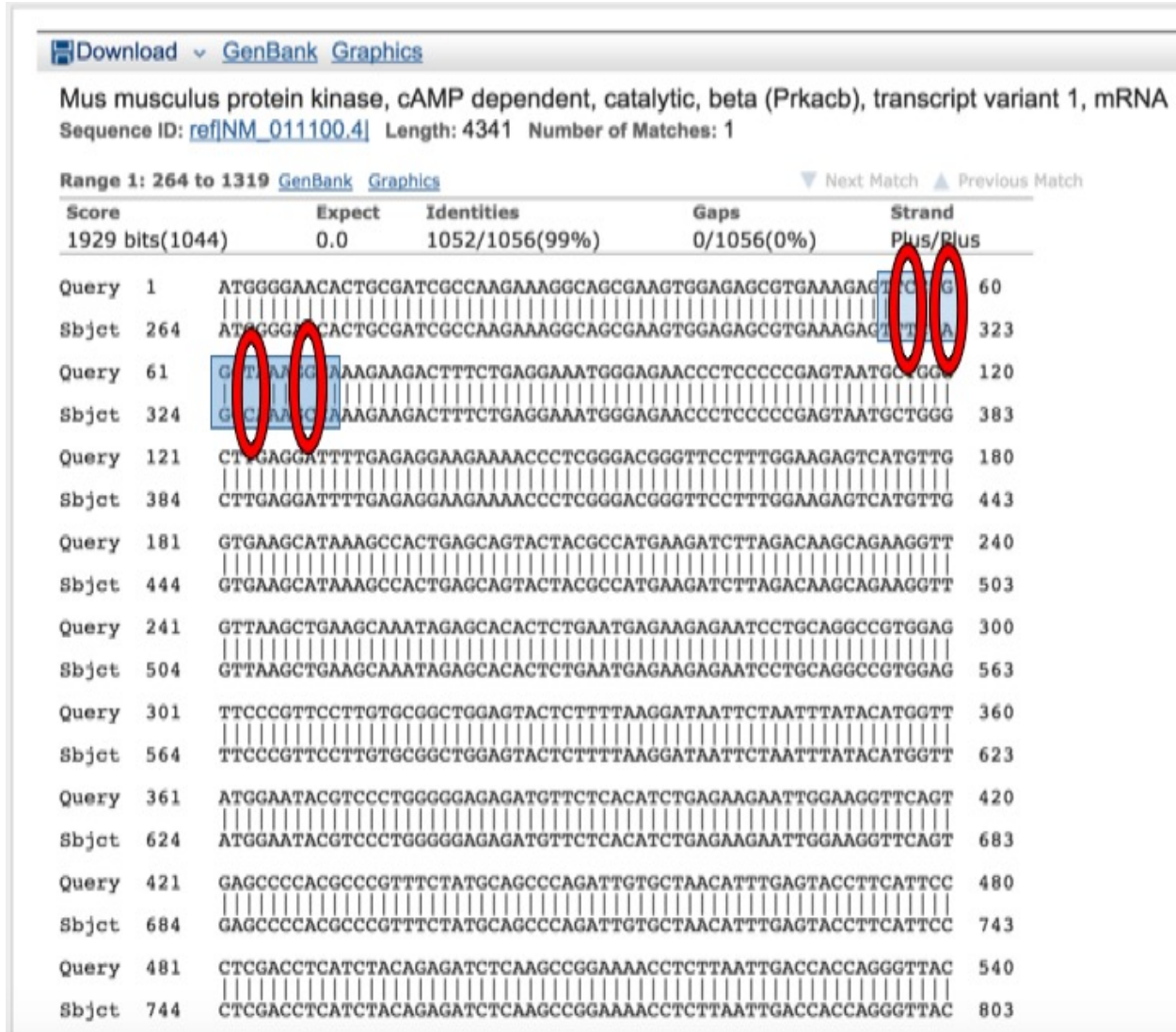
B



**Figure 5: siRNA knockdown and rescue with PKA-C $\alpha$ -YFP plasmid containing one additional mutation (T $\rightarrow$ C).** Cells were plated such that there 100,000 cells per well. Each well received no siRNA, scramble siRNA or  $\alpha\beta$  siRNA. Cells were harvested and then a Western blot was performed. Cells were then transfected with YFP plasmid or YFP-PKAC $\alpha$  plasmid 24 hours after siRNA transfection. A group of cells was not transfected with plasmid DNA as a control. The media was changed the following day, allowing for 48 hours of exposure to the siRNA. Cells were harvested 48 hours after the plasmid transfection to allow for enough time to express the plasmid. A) Fluorescent microscopy was used to verify transfection efficiency prior to Western blotting. B) 10ug of each sample was loaded into the gel. Tubulin was used as a loading control.

Figure 8: Sequence alignment to verify the introduction of siRNA silent point mutations into PKA-Cβ.

A



B

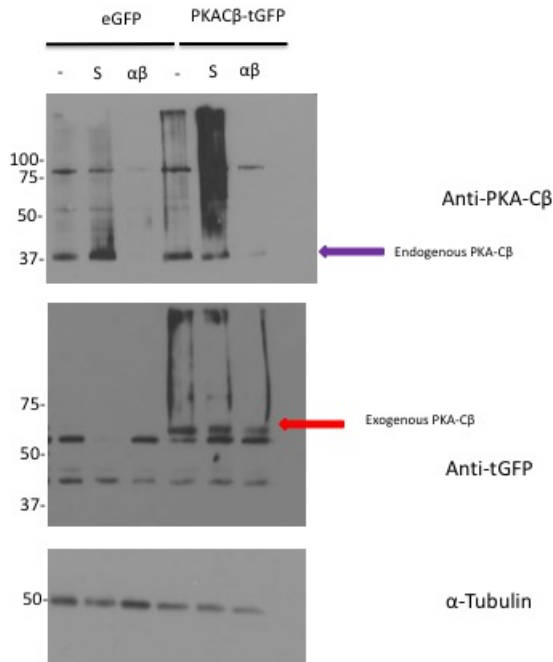
Mus musculus protein kinase, cAMP dependent, catalytic, beta (Prkacb), transcript variant 1, mRNA  
 Sequence ID: [ref|NM\\_011100.4](#) Length: 4341 Number of Matches: 1

Range 1: 264 to 1319 [GenBank](#) [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1923 bits(1041)	0.0	1051/1056(99%)	0/1056(0%)	Plus/Plus
Query 1	ATGGGGAACACTGCGATCGCCAAGAAAGGCAGCGAAGTGGAGAGCGTGAAAGACTTCGG			60
Sbjct 264	ATCGGGAACACTGCGATCGCCAAGAAAGGCAGCGAAGTGGAGAGCGTGAAAGACTTCGG			323
Query 61	GTTAAGCAAAGAAGACTTCTGAGGAAATGGGAGAACCCTCCCCGAGTAATGCTGGG			120
Sbjct 324	GTTAAGCAAAGAAGACTTCTGAGGAAATGGGAGAACCCTCCCCGAGTAATGCTGGG			383
Query 121	CTTGAGGATTTTGGAGAGGAAGAAAACCTCGGGACGGGTTCTTTTGAAGAGTCATGTTG			180
Sbjct 384	CTTGAGGATTTTGGAGAGGAAGAAAACCTCGGGACGGGTTCTTTTGAAGAGTCATGTTG			443
Query 181	GTGAAGCATAAAGCCACTGAGCAGTATACGCCATGAAGATCTTAGACAAGCAGAAGGTT			240
Sbjct 444	GTGAAGCATAAAGCCACTGAGCAGTATACGCCATGAAGATCTTAGACAAGCAGAAGGTT			503
Query 241	GTTAAGCTGAAGCAAATAGAGCACACTCTGAATGAGAAGAGAATCTGCAGGCCGTTGGAG			300
Sbjct 504	GTTAAGCTGAAGCAAATAGAGCACACTCTGAATGAGAAGAGAATCTGCAGGCCGTTGGAG			563
Query 301	TTCCCGTTCCTTGTGCGGCTGGAGTACTCTTTTAAGGATAATTCTAATTTATACATGGTT			360
Sbjct 564	TTCCCGTTCCTTGTGCGGCTGGAGTACTCTTTTAAGGATAATTCTAATTTATACATGGTT			623
Query 361	ATGGAATACGTCCCTGGGGGAGAGATGTTCTCACATCTGAGAAGAATTGGAAGGTTTCAGT			420
Sbjct 624	ATGGAATACGTCCCTGGGGGAGAGATGTTCTCACATCTGAGAAGAATTGGAAGGTTTCAGT			683
Query 421	GAGCCCCACGCCCGTTTCTATGCAGCCAGATTGTGCTAACATTTGAGTACCTTCATTCC			480
Sbjct 684	GAGCCCCACGCCCGTTTCTATGCAGCCAGATTGTGCTAACATTTGAGTACCTTCATTCC			743
Query 481	CTCGACCTCATCTACAGAGATCTCAAGCCGGAAAACCTCTTAATTGACCACCAGGGTTAC			540
Sbjct 744	CTCGACCTCATCTACAGAGATCTCAAGCCGGAAAACCTCTTAATTGACCACCAGGGTTAC			803
Query 541	ATCCAGGTCACAGATTTCCGGTTCGCCAAAAGAGTCAAGGGCAGGACATGGACATTGTGT			600
Sbjct 804	ATCCAGGTCACAGATTTCCGGTTCGCCAAAAGAGTCAAGGGCAGGACATGGACATTGTGT			863

**Figure 6: Sequence alignment verifying the introduction of the siRNA mutations and the Y69F mutation by BioBasic using BLAST.** BioBasic sequences were aligned using BLAST. Lines labeled “Query” are the sample sequences inputted into the database. The “Sbjct” represents the sequence hit within the database that our sample aligned with. A) The alignment of WT-PKA-C $\beta$  shows that the four silent mutations were successfully introduced (red circles). The siRNA target region is indicated by the blue box B) The alignment of Y69F-PKA-C $\beta$  also shows the four silent mutations (red circles) as well as the Y69 mutation resulting in tyrosine becoming phenylalanine (green circle). The siRNA target region is indicated by the blue box.

Figure 9: Four silent mutations introduced into PKA-C $\beta$  conveyed resistance to siRNA.



**Figure 7: siRNA knockdown and rescue with PKA-C $\beta$ -tGFP plasmid containing one additional mutation as indicated in Table 1.** Cells were plated such that there 100,000 cells per well. Each well received no siRNA, scramble siRNA or  $\alpha\beta$  siRNA. Cells were harvested and then a Western blot was performed. Cells were then transfected with eGFP plasmid or tGFP-PKAC $\beta$  plasmid 24 hours after siRNA transfection. A group of cells was not transfected with plasmid DNA as a control. The media was changed the following day, allowing for 48 hours of exposure to the siRNA. Cells were harvested 48 hours after the plasmid transfection to allow for enough time to express the plasmid. 25  $\mu$ g of each sample was loaded into the gel. Tubulin was used as a loading control.