AD_____

Award Number: W81XWH-10-1-0983

TITLE: Signaling through the PI 3-K, Akt, and SGK Pathway in Breast Cancer Progression

PRINCIPAL INVESTIGATOR: Dr. Sivan Elloul

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, MA 02215

REPORT DATE: October 2012

TYPE OF REPORT: Annual Summary

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
October 2012	Annual Summary	30 September 2011 - 29 September 2012

4. TITLE AND SUBTITLE
Signaling through the PI 3-K, Akt, and SGK Pathway in Breast Cancer Progression

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0983

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Sivan Elloul

E-Mail: selloul@bidmc.harvard.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Beth Israel Deaconess Medical Center
Boston, MA 02215

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

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

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The aggressive behavior of malignant breast cancer is determined by a complex array of signaling pathways that regulate cell growth, survival and migration. The PI 3-K-Akt pathway has been linked to all of these responses. The current paradigm states that deregulated PI 3-K-Akt signaling promotes cancer progression. Many of the enzymes that regulate PI 3-K signaling are frequently mutated in human breast cancer, thereby up-regulating Akt activity and increasing tumor cell growth and survival. This is best illustrated by the finding that the catalytic subunit of PI 3-K, PIK3CA, is the most frequently mutated oncogene in breast cancer. However, recent studies have demonstrated that distinct Akt isoforms can either inhibit or enhance breast cancer cell invasive migration and metastasis in vitro and in vivo, probably by phosphorylating a different set of substrates in an isoform specific manner. Afadin is a Ras target gene that regulates cell-cell adhesion downstream of Ras. Afadin protein loss of expression is associated with poor outcome in breast cancer patients. The most significant finding of this research thus far is that Afadin –isoform 3, which is the longer, ubiquitously expressed form of the protein, is a substrate of Akt, down stream of the PI-3K pathway. This protein translocates to the nucleus following phosphorylation by Akt, which leads to elevated migratory characteristics.

15. SUBJECT TERMS
Phosphorylation, proteomic screen, SILAC

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	15	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents:

Introduction:

Increased Akt activity is detected in aggressive human breast cancers [1, 2] and is associated with poor prognosis and higher probability of relapse accompanied by distant metastases in patients [3]. Experiments in transgenic mice have revealed that Akt promotes mammary tumor progression by increasing cell survival [4]. Moreover, many of the proteins in the Akt pathway are either oncogenes (e.g. Akt itself, PI 3-K, MDM2) or tumor suppressors (e.g. the PIP3 phosphatase PTEN and Akt phosphatase PH domain Leucine-rich repeat Protein Phosphatase (PHLPP)), and indeed these proteins are frequently deregulated in breast cancer. The significance of the Akt pathway in cancer has made it an attractive target for the development of small molecule inhibitors [5]. There are three mammalian Akt isoforms: Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ). The three Akt isoforms are encoded by distinct genes, but share a high degree of amino acid similarity and are activated by similar mechanisms and recognize and phosphorylate the same consensus motif [6]. The objectives of this research are to discover novel substrates of the PI-3K-Akt pathway that promote malignant phenotype in breast cancer, with a special scope on Akt-isoform specific substrates and Afadin, as a putative novel substrate of PI-3K pathway.

In the previous reported I was able to show that Afadin isoform 3 in a novel substrate of all 3 Akt isoforms. The phosphorylation site is S1718, and it is sensitive to a line of PI-3K and Akt inhibitors.

Body:
In the previous year I found that Afadin isoform 3 is a novel substrate of the PI-3K pathway. I found that it is a substrate of Akt. The phosphorylation site is S1718. I was trying to find out whether it's an Akt isoform specific substrate, in this part I found that it's a general substrate that can be phophorylayted by all three Akt isoforms but not by other AGC kinases.
In the reported year I was focusing on trying to establish the biological significance of this phosphorylation, and to reveal the connection between PI-3K and Akt to this important adhesion protein.
In the previous year I also started the proteomic screen. I had silenced the expression of a specific kinase by the pLKO-Tet-On system. The main goal of this part of the research is to find novel isoform specific substrates of Akt that would help explain the importance of this family of kinases in many processes in breast cancer.

Description of the research accomplishment associated with the tasks outlined in the approved statement of work:
Aim1:Afadin Regulates Breast Cancer Cell Migration Downstream of Akt and SGK.
Aim1 task 2: Explore the functional consequence of Afadin-i3 phosphorylation by Akt and SGK signaling in breast cancer cells. (timeframe months 12-18)
Subtask 2a: Transwell migration assays. (timeframe months 12-15)
Using T47D cells who do not express endogenous AF6 I was able to explore the role of the S1718 phosphorylation site in migratory behavior of these aggressive cells. I generated several mutants to the phosphorylation site.
Mutation of the Serine in 1718 to Alanin (S1718A), this mutant is a non-phosphorylatalbe mutant. It would not be phosphorylated under any condition.
Mutation of the Serine to Aspartic Acid or Glutamic Acid (S1718D or S1718E), these mutants posses high negative charge that makes them "Phosphomimetic",that means that these mutants serve as a positive control for phosphorylation, or mimic the phosphorylated protein state under all conditions.
These mutants were transfected to T47D cells and the cells were used for Migration assay (Figure 1)

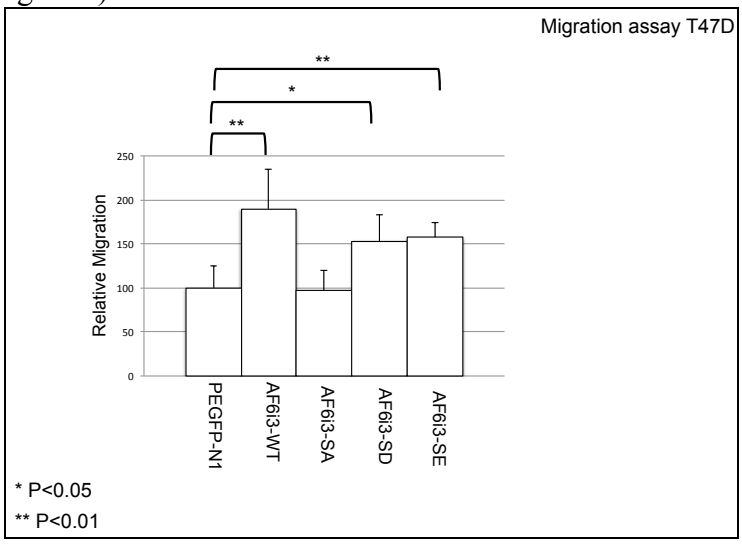

Figure 1:
Migration of T47D cells

Cells were also transfected with Vector empty control (pEGFP-N1). The results clearly show that the cells transfected with the non-phosphorylatable allele migrate as good as the Vector control cells. On the other hand, cells that were transfected with Wild type AF6i3 (WT) or SD or SE mutants migrate significantly better. The only difference between the plasmids that were used to transfect the cells is the specific mutant that was introduced in the 1718 position. Figure 1 is a summary of 3 independent experiments that were done each in triplicates.

Subtask 2b: Matrigel invasion assays. (timeframe months 12-15). I was trying to do invasion assays on several cell lines including T47D. Unfortunately, this assay did not work so well in my hands. The difference in migration that was achieved in Figure 1 was following an incubation of 16 hours. Invasion assay is quite similar to migration assay, with one big difference, the wells are coated with Matrigel, which makes the invasion through this layer, a more challenging process for the cells. In my experiments I wasn't able to see enough cells after invasion assay in order to make a conclusion about the processes that these cells are going through.

Subtask 2c: shRNA rescue experiments using mutated protein. (timeframe months 15-18)

The goal of this subtask was to a reveal the genuine role of the phosphorylation site in the biological context. In this set of experiments I silenced endogenous AF6i3 with shRNA lentivirus, and then over expressed the different mutants (as non-silenceabale alleles). I then did Immunofluorescence in order to check intra-cellular localization and if it changes as a result of the phosphorylation. In Figure 2 we can see that in MCF10A cells endogenous AF6 is membranal and there is some nuclear staining as well. The antibody that was used here for AF6 detects all isoforms. Following specific shRNA that targets AF6i3 alone the signal is abolished, which means that the main isoform of AF6 in these cells is isoform 3. We can also see that under all conditions the phosphorylated form of the protein (which is detected by pAF6 antibody) is nuclear. In the rescue experiment, when the wild type protein is introduced, a mix of membrane and nuclear signal is seen, whereas in the case of the SA mutant (non-phosphorylateable allele) the signal is membranal. In the case of the phosphomimetic alleles (SD and SE) the signal is only nuclear. These results suggest that the phosphorylation of S1718 leads to the nuclear localization of this big protein.

To check whether this is also applicable for endogenous protein under IGF stimulation, I continued by treating the cells with IGF for long periods of time. In Figure 3, MCF10A cells were serum starved over night and then stimulated with IGF1 for 4 additional hours. The nuclear localization of the phosphor-protein is best seen within the time-frame of 4-6 hours of stimulation with IGF1. We can see that in the cells that were only serum starved, the signal is membranal, whereas in the cells that were also stimulated for 4 hours with IGF1, the signal is more nuclear. In the case of the pAF6 antibody, the nuclear signal is strong after 4 hours of stimulation with IGF1, with no membranal signal.

In order to establish a firm connection between nuclear localization of AF6 and the phosphosite, an experiment with PI-3K inhibitors was conducted. In this experiment (Figure 4) MCF10A cells were serum starved, and then either treated with PI-3K inhibitor (BEZ234, Wortmannin), or P110alpha inhibitor (A66) or Akt inhibitor (MK2206) in addition to stimulation with IGF1 for 4 hours. The results in Figure 4 clearly show that the nuclear localization of AF6 that is achieved by stimulation with IGF1 is abolished upon pre-treatment with these inhibitors, suggesting that the nuclear localization is caused by the phosphorylation of S1718 in Afadin.

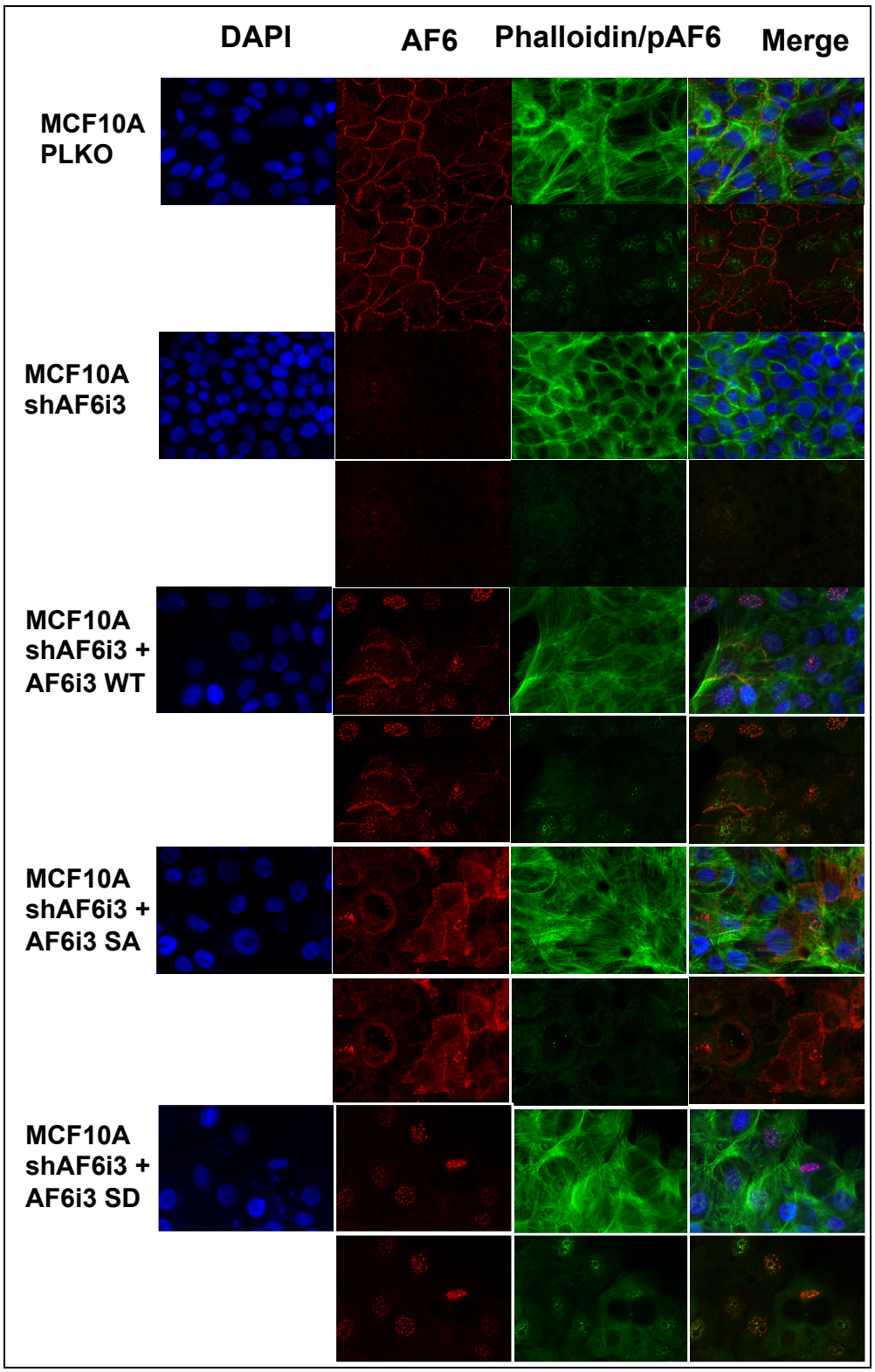

Figure 2:
Immunofluorescence of MCF10A cells in a rescue experiment with AF6i3mutants.

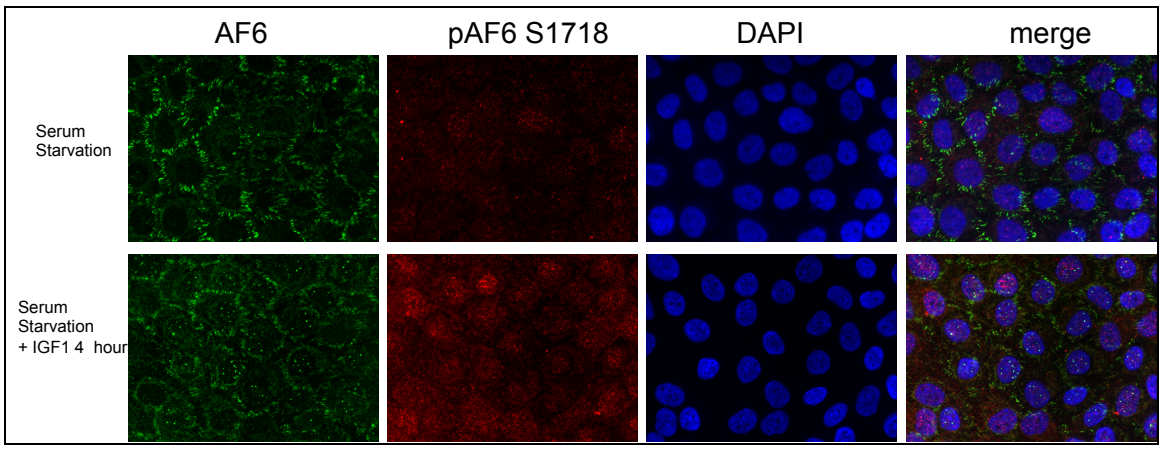

Figure 3:
Immunofluorescence of MCF10A cells under serum starvation or with IGF1 stimulation for 4 hours.

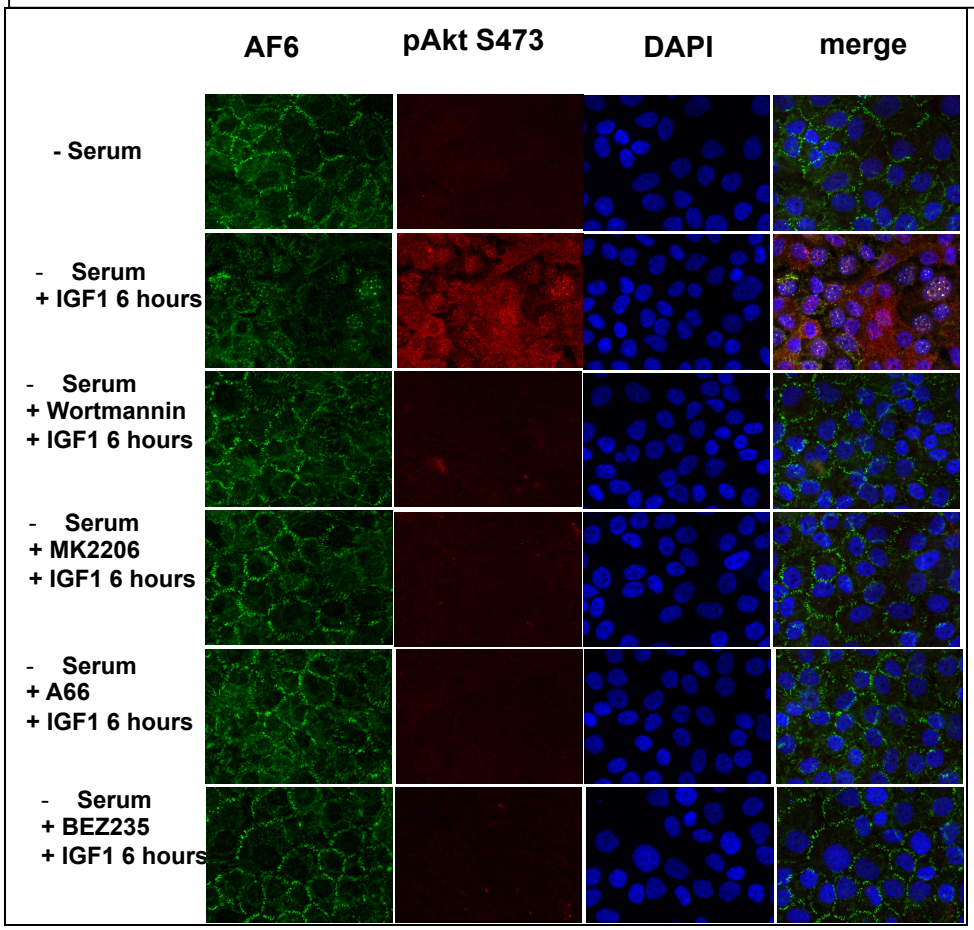

Figure 4:
Immunofluorescence of MCF0A cells following treatment with PI-3K inhibitors and

Subtask 2d: Actin bundling assays. (timeframe months 15-18)
Subtask 2e: Actin binding assays. (timeframe months 15-18)
These two subtasks were explored in a pilot rescue experiment in which endogenous AF6i3 was silenced and the mutants were over expressed. Not surprisingly, there was no difference between the mutants in binding and bundling of Actin. My initial hypothesis was that since the phosphosite is within the actin binding domain, the phosphorylation would affect Actin binding or bundling. The immunofluorescent experiments, in which in a lot of cases I stain with Phaloidin (a toxin that bind to Actin), I saw that there is no difference in the staining between the different mutants, so eventually the biology of this protein took this story to another direction. These days I'm exploring the effects of phosphorylation and nuclear localization on the stability of the protein, and it seems that the nuclear localization of the protein is a way of the cell to stabilize the protein and "escape" from degradation (this preliminary data is not shown here).

Aim 2: Phospho-proteomic analysis of Akt1/2/3 and SGK1/2/3 substrates in breast cancer
Subtask 2c: : Perform Stable Isotope Labeling by Amino acids in Cell culture (SILAC) experiment. (timeframe months 12-15).
As mentioned in last year's report, this subtask was preformed and the cells were sent to our collaborator for Mass Spectrometry.
Subtask 2d: Mass Spectrometry (timeframe months 12-15).
Aim 2 task 3: MS data analysis. (timeframe months 16-18)
The experiment was done using 2 different antibodies for the phosphomotif. Table 1 summarizes some of the results we got.

Protein Name	Site	Silac Ratio (-Dox/+Dox)					
		Tet-on Akt1 shRNA		Tet-on Akt2 shRNA		Tet-on Akt3 shRNA	
		Ab1	Ab2	Ab1	Ab2	Ab1	Ab2
Akt3 Substrates							
Chk1 Known Akt substrate	280	1.04	1.32	1.14	1.05	-1.05	-1.38
Hs2 (ZCCHC6) Implicated in cell cycle checkpoint control	132	1.3	1.03			-2.06	1.16
Akt1 and Akt3 Substrates							
Adam22 Also shown to be phosphorylated by Akt1 in LNCap screen	834		-1.36		1.11	-1.35	-1.24
LMO7 Transcriptional regulator Bind alpha-actinin (aa 796-1076)	805	-1.04	-1.28	1.13	1.38	-1.32	-1.28
EMSY Transcriptional regulator	209	-1.25	-1.35	1.04	1.15	-1.03	1.08

Table 1:
Putative new substrates of Akt isoforms

In order for a protein containing a putative phosphosite to be considered as a "hit", ratios that were received using both antibodies should be negative. That means that following treatment with Doxycyline, which led to the silencing of the specific Akt isoform, phosphorylation was inhibited. If the ratio between treatment to no treatment with Doxycyline is negative for Akt1 for example, but positive for Akt2 and Akt3, it may suggest that this is a phosphosite that can be phosphorylated mainly or solely by Akt1.
Table 1 lists a few novel and known substrates of Akt kinases.
Aim 2 task 4: Select 2 candidates for additional validation as outlined in Aim 1. (timeframe months 19-36)
For this subtask I chose to focus on Adam22. Adam 22 (A Disintegrin and metalloproteinase domain-containing protein 22) is a ligand for integrin in the brain.This is a non catalytic metalloprotease-like protein. Involved in regulation of cell adhesion and spreading and in inhibition of cell proliferation. I chose this protein because of its possible involvement in migration and metastatic processes in cancer. Adam22 is a putative substrate of Akt that according to the results in Table 1 could be phosphorylated by Akt1 and Akt3 but not Akt2. In order to check whether this is correct, an initial experiment was performed in which HeLa cells were stimulated with IGF1 for 20 minutes, or pre-treated with MK2206 (Akt inhibitor) and then stimulated with IGF1 for 20 minutes. Figure 5 shows that Adam22 phosphorylation is stimulated by IGF1 and inhibited by the Akt inhibitor MK2206. HeLa cells were transfected with GFP-Adam22, cells were stimulated with IGF1, or pre-treated with MK2206 and then stimulated with IGF1 for 20 minutes. The cells were harvested and immunoprecipitated with an antibody against the GFP tag. In order to check if the protein is phosphorylated the blot was incubated with an antibody against the Akt phosphorylation motif (RxRxxS/T). The phosphorylation is induced by IGF1 and following MK2206 treatment goes back to the basal level, which indicates that this phosphorylation is Akt dependent.

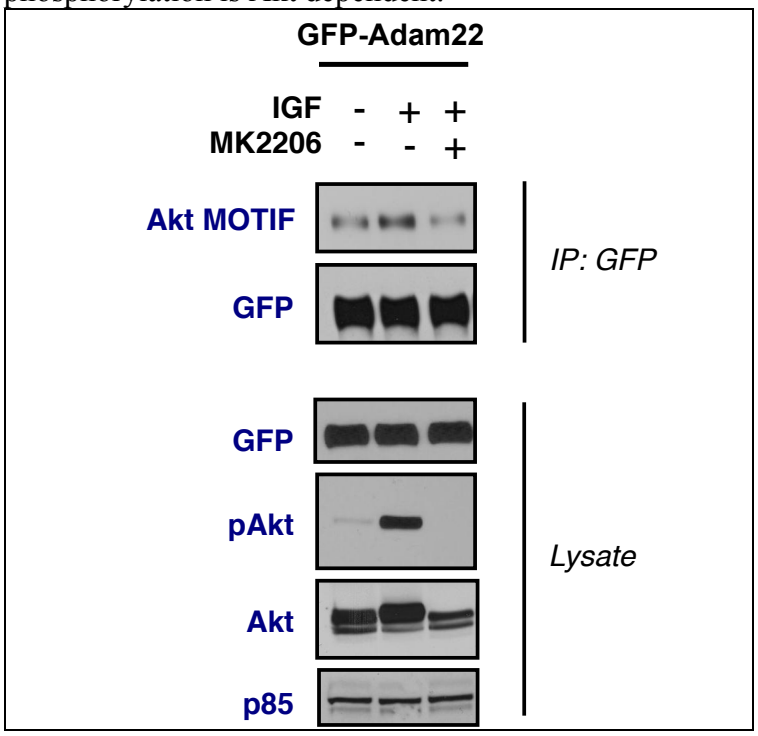

Figure 5:
Western Blot of HeLa cells transfected with GFP-Adam22, treated with IGF1 and Akt inhibitor.

In order to publish my Afadin data, this year I chose to promote my Afadin project and only preliminary data is available on the other putative substrates that were found in the screen.

Key Research Accomplishments:

- Afadin isoform 3 was found to be a novel substrate of Akt in the PI-3K signaling Pathway, that can be phosphorylated in S1718 by all three Akt isoforms.
- The phosphorylation of S1718 leads to a change in intracellular localization of this adhesion protein.
- Following phosphorylation the protein translocates into the nucleus, whereas the non-phosphorylatable protein is only membranal.
- Phosphorylation of Afadin isoform 3 in S1718 leads to enhanced migration of the cells.

Reportable Outcomes:
My training program in the last year included weekly meetings with Dr. Toker, my postdoctoral mentor, where we analyzed results and plan experiments. In addition, our laboratory meets once a week for a lab meeting. In these meetings we receive feedback on our work, as well as advice and input. In the last year I presented my project 6 times in lab meetings. Moreover, the Toker laboratory has a bi-weekly journal club meeting where discussions focus on recently published literature in the breast cancer field. I presented twice in the journal club and participated in all of the meetings we had. Finally, as part of the various intramural training activities within the laboratory, our lab attends a weekly cancer signaling meeting at Harvard Medical School. These meetings are designated to refine students' and postdocs' presentation skills. These meetings allow the participants and speakers to receive feedback on work from leaders in their respective fields, including Lewis Cantley, Joan Brugge and John Blenis. In the reported year I presented once in this forum.

Conclusions:

In the reported year I was trying to find out what are the biological consequences of the phosphorylation of S1718 in Afadin isoform 3. This is a novel substrate of the PI-3K / Akt pathway. I found that following the phosphorylation of S1718 there is a translocation of this big otherwise membranal protein to the nucleus. This is an interesting phenomenon because Afadin is a very big protein, that up until now was thought to reside in the membrane alone. This process of nuclear translocation could have several implications. First, it's possible that the nuclear translocation of Afadin leads to activation of transcription factor or other pathways in the nucleus. Another option is that it's the loss of the membranal signal itself that leads to the "loss of activity" of Afadin as an adhesion protein in the membrane.

In addition, phosphorylation of Afadin isoform 3 in S1718 changes the migratory characteristics of cells. The non-phosphorylatable protein is less migratory than the wild type and the phosphomimetic protein. In other words, in order for the protein to serve as an adhesion protein and "protect" cells from migration, it cannot be phosphorylated. Once the protein is phosphorylated one of 2 things can happen, either the nuclear translocation itself leads to elevation in migration through activation of other pathways or proteins, or the loss of the membranal signal is responsible for the elevated migration of these cells. Since PI-3K and Akt are highly activated in breast cancer, it's possible that Afadin is constantly phosphorylated and hence resides in the nucleus and not activated as an adhesion protein in the membrane.

With the proteomic screen, I have a list of putative substrates of Akt isoforms, some of them are known and some are new and their phosphorylation has to be proved in vitro before making any conclusions. Over all, this approach of silencing a specific kinase to find isoform specific substrates, although not very efficient, but it seems to work fine.

References:

1. Sun, M., et al., *AKT1/PKBalpha kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells.* Am J Pathol, 2001. **159**(2): p. 431-7.
2. Staal, S.P., *Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma.* Proc Natl Acad Sci U S A, 1987. **84**(14): p. 5034-7.
3. Perez-Tenorio, G. and O. Stal, *Activation of AKT/PKB in breast cancer predicts a worse outcome among endocrine treated patients.* Br J Cancer, 2002. **86**(4): p. 540-5.
4. Hutchinson, J., et al., *Activation of Akt (protein kinase B) in mammary epithelium provides a critical cell survival signal required for tumor progression.* Mol Cell Biol, 2001. **21**(6): p. 2203-12.
5. Hennessy, B.T., et al., *Exploiting the PI3K/AKT pathway for cancer drug discovery.* Nat Rev Drug Discov, 2005. **4**(12): p. 988-1004.
6. Woodgett, J.R., *Recent advances in the protein kinase B signaling pathway.* Curr Opin Cell Biol, 2005. **17**(2): p. 150-7.
7. Vasudevan, K.M., et al., *AKT-independent signaling downstream of oncogenic PIK3CA mutations in human cancer.* Cancer Cell, 2009. **16**(1): p. 21-32.