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TITLE:

TNK2 Tyrosine Kinase as a Novel Therapeutic Target in Triple-Negative Breast Cancer

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<b>14. ABSTRACT</b> Triple-negative breast cancers (TNBCs) represent only 10%-15% of all breast cancers; however, they are highly aggressive and have a higher rate of metastasis. In order to explore the role of tyrosine kinase signaling in TNBCs, we have performed global phosphotyrosine profiling for a panel of 25 TNBC cell lines. When we correlated protein phosphorylation levels with cellular oncogenic phenotypes, we observed a novel non-receptor tyrosine kinase, TNK2, to be hyperphosphorylated and activated in highly aggressive TNBC cells. Suppression of TNK2 by specific siRNAs significantly reduced the proliferation, colony formation, and invasive ability of TNBC cells. The objective of this proposal is to evaluate the therapeutic potential of TNK2 in the treatment of TNBCs. The Specific Aims of this project are: Aim 1: Do TNK2 protein levels and activation correlate with clinical and pathological features of TNBC? Aim 2: What is the value of TNK2 as a therapeutic target in vitro and in preclinical animal models? Aim 3: How is TNK2 signaling altered during oncogenesis in TNBCs?					
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## **INTRODUCTION:**

Triple negative breast cancers (TNBC) represent 10-15% of all breast cancers. While significant advances have been made for targeted therapy of ER and HER2-positive breast cancer patients, systematic chemotherapy is the primary modality used for treatment of TNBC patients as there are no clearly defined TNBC-specific therapeutic targets (1). This proposal aims to revolutionize TNBC treatment regimens by replacing current drugs that have life-threatening toxicities with safer, more effective intervention strategies. By seeking to identify therapeutic targets in primary TNBC tumors at presentation, another overarching challenge limiting patient care that this proposal will address is the high mortality associated with metastatic breast cancer. As part of the preliminary efforts described here, we have performed a comprehensive quantitative phosphoproteomic analysis on a large panel of TNBC-derived cell lines. In this functional screen, we discovered TNK2, also known as ACK1, a non-receptor protein tyrosine kinase, was selectively activated in highly aggressive TNBC cells. In this study, we propose extending our proteomic and biochemical studies to evaluate the therapeutic potential of targeting TNK2 for inhibition in TNBCs using a novel TNK2 small molecule inhibitor, DZ1-067, also known as (R)-9b developed by the Mahajan Lab. In parallel, we will also use proteomic approaches to identify downstream signaling pathways that are specifically modulated upon TNK2 inhibition. Success of the proposed studies will lay a strong foundation to translate our preclinical discovery into a personalized clinical trial for women with TNK2 positive TNBCs and to eventually reduce the mortality rate of at least a subset of TNBC patients.

**KEYWORDS:** TNK2, Triple Negative Breast Cancer, Proteome, Phosphorylation, Mass Spectrometry, Signaling

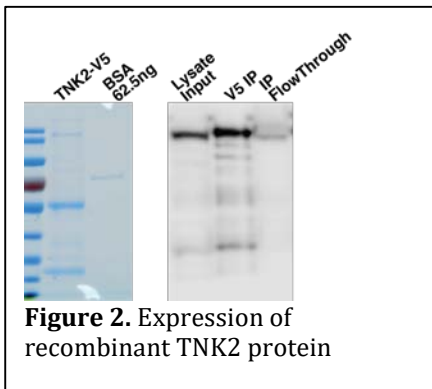
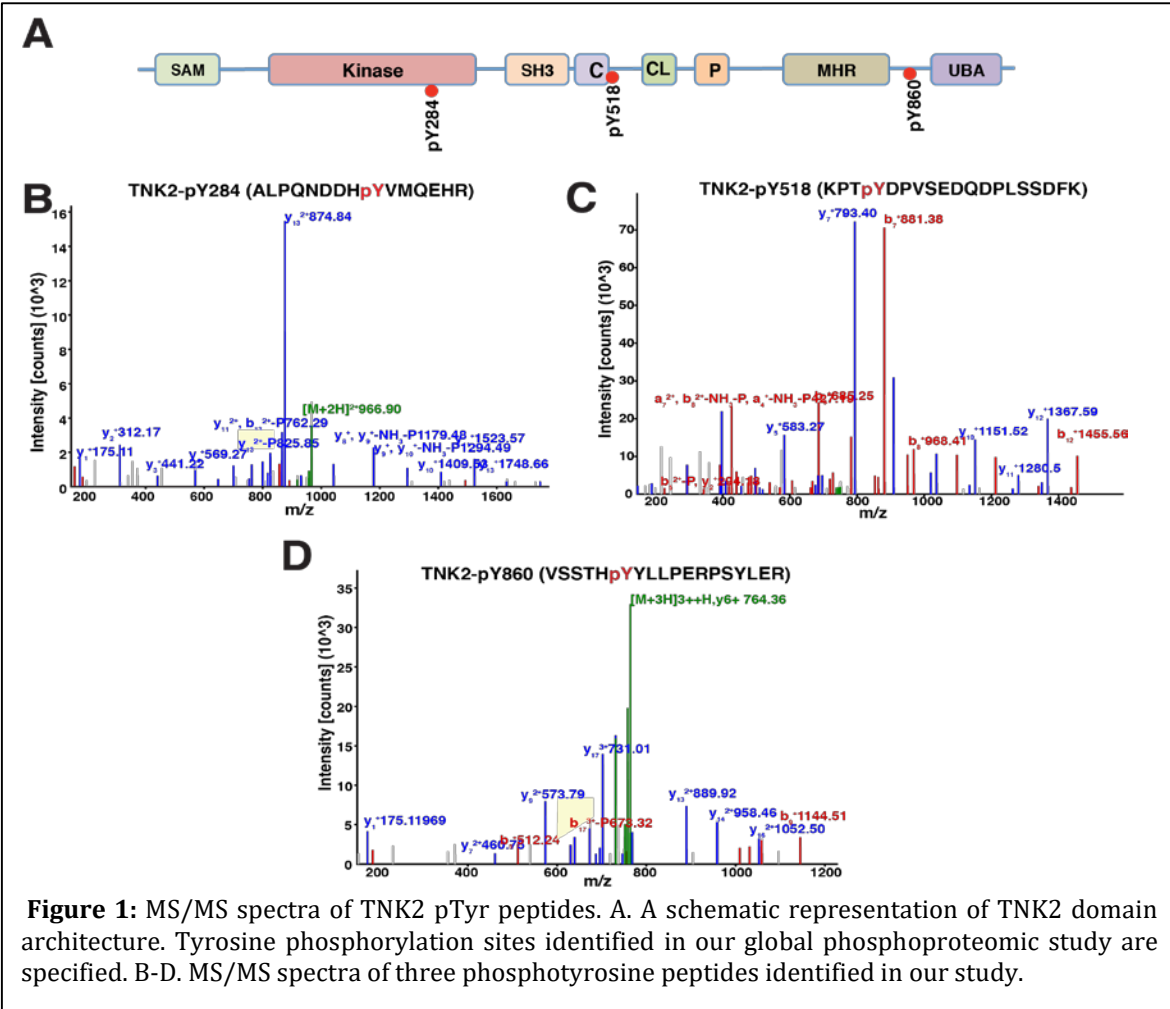
## ACCOMPLISHMENTS:

The major goals of the project in year 2 were as follows:

### What was accomplished under these goals

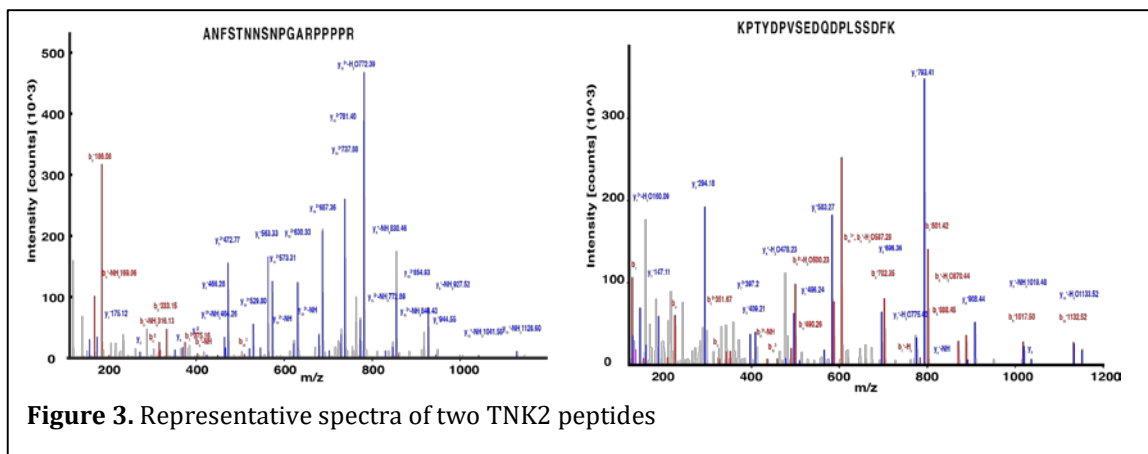
Subtask 1: To select TNK2 peptides and phosphopeptides that can be detected by MRM and optimize the MS/MS methods

### Completed



During the first year of this study, we identified three phosphopeptides of TNK2 and developed MS based PRM analysis to monitor the level of TNK2 phosphorylation (Figure 1). In addition to TNK2 phosphorylation levels, it is also important to investigate the total protein expression of TNK2 in breast cancers. In order to also assess the expression level of TNK2 protein with MS based PRM analysis, we first generated recombinant TNK2-V5 fusion protein in HEK293T cells. The TNK2-V5 proteins

were purified immune affinity enrichment with agarose conjugated anti-V5 antibody. The purified proteins were first subjected by SDS-PAGE followed by Coomassie blue staining (Figure 2 Left panel). We also performed western blot to confirm the correct expression of TNK2 (Figure 2 right panel). The purified TNK2 protein were then digested by trypsin and analyzed by mass spectrometer. In this study, we identified more than 30 TNK2 peptides that cover 39% of TNK2 proteins. Representative MS/MS spectra were shown in figure 3.



Subtask 2: To perform MRM analysis with TNBC breast cancer tissue specimens. 13-15

We have developed robust MS based PRM methods to quantitatively detect TNK2 peptides and phosphopeptides. The protein lysates from TNBC cell lines and breast cancer tissues have been collected. Proteins from these specimens have been trypsin digested and reverse phase-desalted. We have also initiated the PRM analysis to assess the TNK2 levels in these specimens. However, because of the low level phosphorylation of TNK2 in tumor tissues, we now need to enrich TNK2 protein and/or phosphotyrosine peptides to increase the detection sensitivity of our method. We will redo these assays.

Subtask 3: Data acquisition and statistic analysis 16-18

*Milestone #1: Co-authors work on development of a manuscript by compiling the data together 16-18*

### Completed

We have published our discovery in Oncotarget in 2017.

Xinyan Wu, Muhammad Saddiq Zahari, Santosh Renuse, Mustafa A. Bharbuiya, Pamela L. Rojas, Dhanashree S. Kelkar, Vered Stearns, Edward Gabrielson, Pavani Malla, Saraswati Sukumar, Nupam P. Mahajan\* and Akhilesh Pandey

Title: The non-receptor tyrosine kinase TNK2/ACK1 is a novel therapeutic target in triple negative breast cancer.

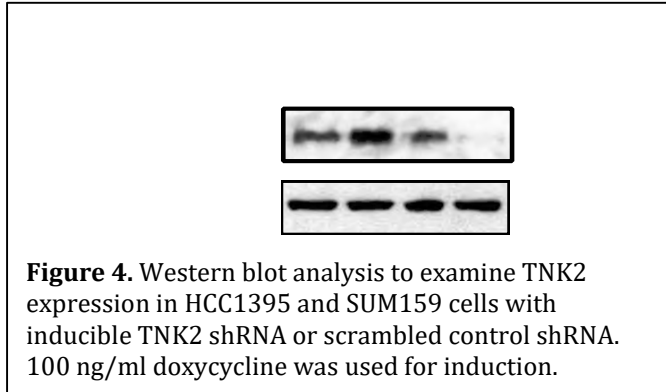
Year: 2017

Journal: *Oncotarget* 17, 8: 2971–2983.

**Specific Aim 2:** What is the value of *TNK2* as a therapeutic target *in vitro* and in preclinical animal models?

**Major Task 1:** To examine oncogenic significance of *TNK2* by suppressing *TNK2* in TNBC cell lines.

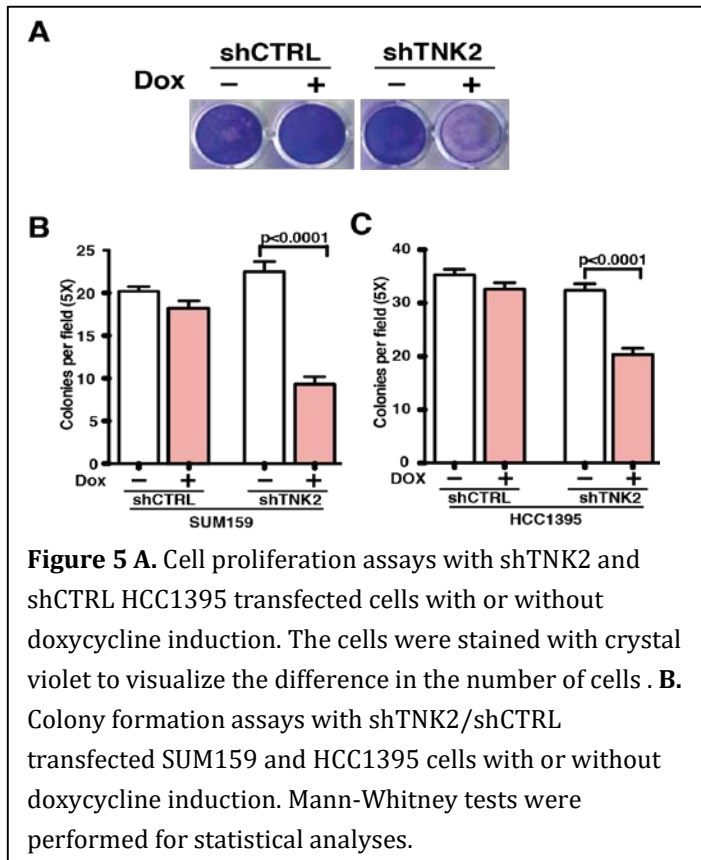
**Subtask 1: 1.** To establish *shTNK2* inducible TNBC cell lines. (12-14)



### Completed

We have established *shTNK2* inducible cell lines using HCC1395 and SUM159 TNBC cells. **Figure 4** demonstrated effective shRNA knockdown of *TNK2* expression in both HCC1395 and SUM159 cells.

**Subtask 2:** To suppress *TNK2* expression by transient transfection of *siTNK2* or induction of *shTNK2* in selected TNBC cell lines. And to perform functional assays to evaluate the role of *TNK2* in regulation of oncogenic phenotypes of TNBC cells. 15-18



### Completed

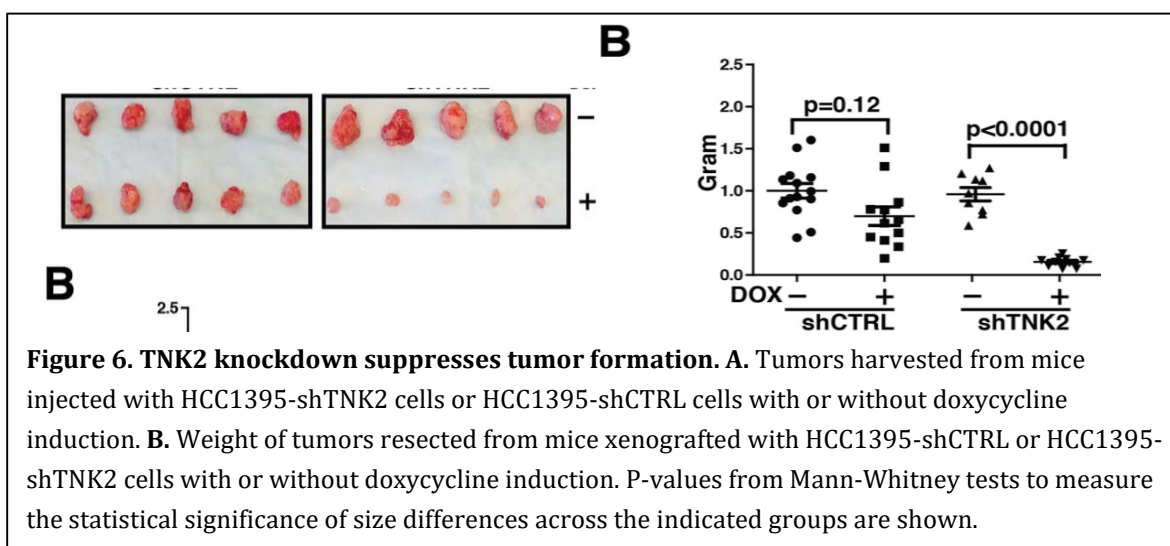
We performed proliferation and colony formation assays to assess the functional role of *TNK2* in TNBC. We observed significant reduction of cell proliferation and colony formation of TNBC cells with suppression of *TNK2* expression (**Figure 5**)

**Major Task 2:** To evaluate therapeutic potential of targeting TNK2 in preclinical animal models.

**Subtask 1:** To perform orthotopic xenograft with shTNK2 inducible TNBC cells and monitor tumor growth of shTNK2 induced and not induced groups. Inducible scramble shRNA (shCTRL) cells will be used as control - 19-26

### Completed

We performed orthotopic xenograft using shTNK2 HCC1395 in NSG mice. We found that induction of shTNK2 expression with doxycycline to knock down the expression of TNK2 significantly reduce the tumor formation in xenograft mouse models. (Figure 6)



**Specific Aim 3:** How does TNK2 signaling change during the oncogenesis of TNBCs?

**Major Task 1:** To map signaling networks in TNBC cells with the suppression of TNK2 expression/activity.

**Subtask 1:** To perform SILAC amino acid labeling for shTNK2 inducible TNBCs and wild type TNBC cells 20-21

### Completed.

We have labeled the shTNK2 cells for more than 5 passages. Mass spectrometry analysis was performed to confirm >95% labeling efficiency was achieved (data not shown).

**Subtask 2.** To expand cell culture for SILAC heavy, medium and light labeled TNBC cells for phosphoproteomic analysis 22-24

### Completed



We have expanded cell culture for SILAC heavy medium and light labeled TNBC cells for more than 15-cm dishes per state. Cells were lysed with 9 M urea lysis buffer.

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

This project provided an opportunity for training and professional development to Dr. Xinyan Wu. Dr. Wu is an Instructor in the Department of Biological Chemistry. Dr. Wu participated in the project and performed the mass spectrometry analysis, western blotting and cell functional assays.

**How were the results disseminated to communities of interest?**

Based on this study and our previous proteomics data, we published a manuscript in the journal *Oncotarget*. This was attached in last year's update as an accepted manuscript, which has since been published.

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

We plan to perform experiments described in Specific aim 3 Major task 1 and Major task 2 described briefly below:

**Specific Aim 3:** How does TNK2 signaling change during the oncogenesis of TNBCs?

**Major Task 1:** To map signaling networks in TNBC cells with the suppression of TNK2 expression/activity.

Subtask 1: To perform SILAC amino acid labeling for shTNK2 inducible TNBCs and wild type TNBC cells

Subtask 2: To expand cell culture for SILAC heavy, medium and light labeled TNBC cells for phosphoproteomic analysis

Subtask 3: To perform anti-pTyr antibody based pTyr peptides enrichment followed LC-MS/MS analysis

Subtask 4: To perform TiO<sub>2</sub> based pSer/Thr peptides enrichment followed LC-MS/MS analysis

**Major Task 2:** To identify downstream signaling modules regulated by TNK2.

Subtask 1: To perform database search using Mascot and Sequest search engines and phosphopeptide will be quantified using Proteome Discoverer

Subtask 2. 2. To perform bioinformatic analysis to identify signaling molecules and networks regulated by TNK2

**IMPACT:**

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

Breast cancer is the most prevalent cancer in women worldwide. About 15-20% of all breast cancers do not express estrogen receptor, progesterone receptor or HER2 receptor and hence are commonly classified as triple negative breast cancer (TNBC). These tumors are often relatively aggressive when compared to other types of breast cancer, and this issue is compounded by the lack of effective targeted therapy. In our phosphoproteomic profiling effort, we identified the non-receptor tyrosine kinase TNK2 as activated in a majority of aggressive TNBC cell lines. In our studies, we have demonstrated that TNK2 is overexpressed and hyperphosphorylated in aggressive TNBC cells. Suppression of TNK2 using specific inducible shRNA could significantly inhibit cell proliferation and anchorage independent growth of TNBC cells in vitro. This shRNA-based expression-knockdown could also dramatically reduce tumor formation in xenograft mouse models. Our data suggest that TNK2 has potential to be developed a novel therapeutic target to treat patients with TNBC.

**What was the impact on other disciplines?**

Nothing to Report.

**What was the impact on technology transfer?**

Nothing to Report.

**What was the impact on society beyond science and technology?**

Nothing to Report.

**CHANGES/PROBLEMS:**

**Changes in approach and reasons for change**

Some of the TNBC cell lines such as HS578T and HCC1806 grew very slowly and the infection efficiency of shRNA lentivirus for these cells is low. We have had problems to get stable shRNA expression clones after selection. We will continue to try this in year three.

**Changes that had a significant impact on expenditures**

None

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

No changes were made in use or care of vertebrate animals, or use of biohazards and/or select agents.

**PRODUCTS:**

Journal publications.

Authors: Xinyan Wu\*, Muhammad Saddiq Zahari, Santosh Renuse, Mustafa A. Bharbuiya, Pamela L. Rojas, Dhanashree S. Kelkar, Vered Stearns, Edward Gabrielson, Pavani Malla, Saraswati Sukumar, Nupam P. Mahajan\* and Akhilesh Pandey\*

Title: The non-receptor tyrosine kinase TNK2/ACK1 is a novel therapeutic target in triple negative breast cancer

Journal: Oncotarget

Year: 2017

**Acknowledgement of federal support: Yes**

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

Nothing to Report.

**Other publications, conference papers, and presentations.**

Nothing to Report.

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

Nothing to Report.

**Technologies or techniques**

Nothing to Report.

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

Nothing to Report.

**Other Products**

Nothing to Report.

**PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?

**Name: Akhilesh Pandey**

Project Role: PI

Researcher Identifier (e.g. ORCID ID): [orcid.org/0000-0001-9943-6127](https://orcid.org/0000-0001-9943-6127)<sup>[1]</sup><sub>[SEP]</sub>

Nearest person month worked: 1.2

Contribution to Project: Dr. Pandey has designed and planned experiments. Dr. Pandey also oversaw the overall progress of the project.

Funding Support: Nothing to report.

**Name: Xinyan Wu**

Project Role: Instructor

Researcher Identifier (e.g. ORCID ID): 0000-0002-7745-1614

Nearest person month worked: 7

Contribution to Project: Dr. Wu has helped Dr. Pandey in planning and performing experiment.

Funding Support: Nothing to report.

**What other organizations were involved as partners?**

This is a Breakthrough Award, Funding Level 2 with Partnering PI, Dr. Nupam Mahajan.

The details of partnering PI and institution are as follows:

Organization Name: Moffitt Cancer Center

Location of Organization: Tampa, FL 33612, U.S.A

Partner's contribution to the project: Partnering institution will perform a distinct set of experiment and submit their own report.

Financial support: None

In-kind support: Partnering PI will perform some of the experiments described in the proposal

Facilities: No facilities of Partnering institution are used so far.

Collaboration: Partnering institution will perform a distinct set of experiment and submit their own report.

Personnel exchanges: None of our personnel are currently working in Partnering institution.

Collaboration: Partnering institution has performed a distinct set of experiments and submitted their own report.