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TITLE: Novel Functions of EZH2 in Triple-Negative Breast Cancer: Translation Into New Biomarker and Treatment Strategies

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b>  In the first year of funding we have been able to characterize the protein-protein interactions (PPIs) between EZH2 and p38 $\alpha$ and with AKT1. We successfully determined the kinetic and binding affinity of these PPIs using recombinant full length proteins. These results confirmed the direct interactions between EZH2 and p38 $\alpha$ and AKT1 as well as the co-immunoprecipitations studies which showed that EZH2 binds to p38 $\alpha$ in both the nucleus and the cytoplasm of MDA-MB-231 cells. We will continue with biochemical and biophysical studies towards mapping the binding site and identifying the key residues essential for these PPIs.					
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## 1. INTRODUCTION

This is the first annual report for a project that aims to understand the non-canonical functions of EZH2 as a determinant of breast cancer invasion and metastasis, and to elucidate the relevance of cytoplasmic expression of EZH2. EZH2 (Enhancer of Zeste Homolog 2), a Polycomb group protein presumed to function in controlling the transcriptional memory of a cell [1], is up-regulated during progression from ductal carcinoma in situ, the precursor of invasive carcinoma, to invasive carcinoma and distant metastasis [2]. Furthermore, EZH2 protein is over-expressed in 55% of invasive breast carcinomas, and is significantly associated with poorly differentiated tumors [2-4]. We have previously shown that EZH2 is a powerful independent prognostic biomarker in breast cancer, providing outcome information above and beyond conventional prognosticators used in the clinical setting [2]. We have also demonstrated by Kaplan-Meier analysis that tumors with high EZH2 expression had a worse disease free and overall survival than tumors with low EZH2 expression. Our recent studies support that EZH2 is expressed in the cytoplasm in aggressive breast carcinomas from Ghanaian women [5]. Furthermore, we have found that EZH2 binds to p38 and AKT1 in breast cancer cells, leading to their methylation. These data led us to hypothesize that EZH2 is expressed in the cytoplasm of a subset of TNBC tumors where it methylates and activates p38 and AKT1 leading to metastasis. We further hypothesize that detection of cytoplasmic EZH2, phosphorylated p38 and/or phosphorylated AKT1 proteins may identify TNBC tumors with more aggressive behavior and heightened metastasis.

In this report we are summarizing our progress in the first year of funding.

2. **KEYWORDS:** triple negative breast cancer, EZH2, Protein-protein interactions, African, health disparities.

## 3. ACCOMPLISHMENTS

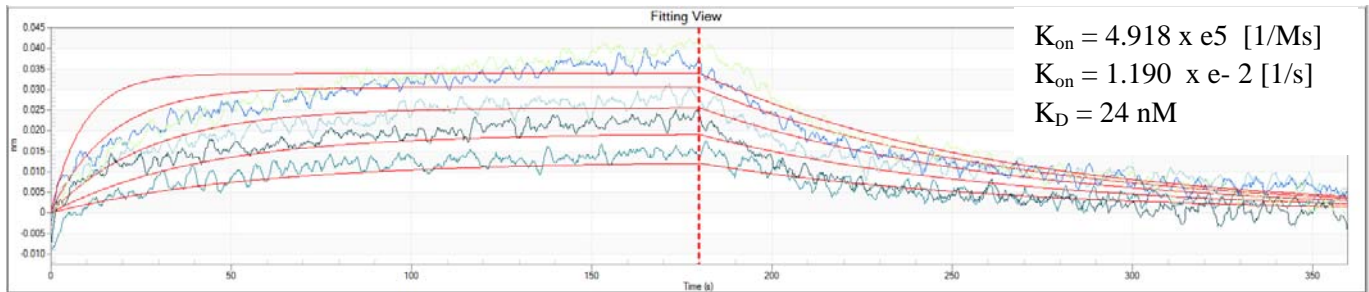
Below are brief descriptions of key accomplishments according to the approved statement of work for Year 1.

**Aim 2: Biochemical and biophysical characterization of the EZH2 interaction with p38 and AKT1 proteins and mapping EZH2 binding site that interacts with p38 and AKT1.**

Task 3: Determine the biochemical features of the EZH2 binding to p38 and AKT1 (Years 1-2).

During the first year we were able to quantitatively determine the binding affinities of EZH2 protein-protein interactions involved with p38 and AKT1. For this purpose we used full length recombinant proteins. To determine the direct binding and kinetic of the interactions we used bio-layer interferometry method. For this purpose EZH2 protein was successfully biotinylated and immobilized on streptavidin sensors and used for testing the binding of p38 and AKT1 and determine the kinetics of these PPIs. The obtained results showed that EZH2 has significantly higher binding affinity to p38 with  $K_D$  of 24 nM (Figure 1). Importantly, the obtained results confirmed the co-immunoprecipitation studies which

detected endogenous EZH2-p38 interaction in the nucleus and in the cytoplasm of MDA-MB-231 TNBC cells. In contrast, EZH2 binds AKT1 with 14 fold decreased binding affinity ( $K_D = 326$  nM).



**Figure 1. Binding and kinetic data of the PPIs between EZH2 and p38 determined by bio-layer interferometry method.** EZH2 protein was immobilized on the streptavidine sensors and p38 was tested in following concentrations: 12 nM, 25 nM, 50 nM, 100 nM and 200 nM. The binding curves were fitted using 1:1 Langmuir model.

### Future studies

Because of the stronger binding affinity of EZH2 protein to p38 in the next year we will focus on understanding the interaction sites between these two proteins. This is further supported by the importance of p38 signaling as a major promoter of breast tumorigenesis. We will prepare sets of recombinant protein deletion variants to determine the minimum region in EZH2 and p38T1 required for these PPIs. The binding affinity constants will be confirmed by applying surface plasmon resonance analysis. Once this is accomplished, we will generate a series of point mutations that will be tested in binding assays to identify the key residues involved in the interaction and map the minimum fragments required for high affinity interaction. Based on these results we will synthesize peptides to further confirm the interactions and through applying alanine scanning mutagenesis strategy we will be able to determine the nature of these PPIs, electrostatic and/or hydrophobic interactions.

### References

1. Laible, G., et al., *Mammalian homologues of the Polycomb-group gene Enhancer of zeste mediate gene silencing in Drosophila heterochromatin and at S. cerevisiae telomeres.* Embo J, 1997. **16**(11): p. 3219-32.
2. Kleer, C.G., et al., *EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells.* Proc Natl Acad Sci U S A, 2003. **100**(20): p. 11606-11.
3. Bachmann, I.M., et al., *EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast.* J Clin Oncol, 2006. **24**(2): p. 268-73.
4. Collett, K., et al., *Expression of enhancer of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer.* Clin Cancer Res, 2006. **12**(4): p. 1168-74.
5. Pang, J., et al., *Invasive breast carcinomas in Ghana: high frequency of high grade, basal-like histology and high EZH2 expression.* Breast Cancer Res Treat, 2012. **135**(1): p. 59-66.

**Thus, the key research accomplishments in this year of work are:**

- Prepare tools to run direct binding studies including biotinylated EZH2 and immobilizing SSA sensors

- Determined the kinetic parameters,  $k_{on}$  and  $k_{off}$ , of the PPIs between EZH2 and p38 and with AKT1.
- Determined the  $K_D$  of investigated PPIs: EZH2 – p38 and EZH2-AKT1.

#### **4. IMPACT**

For the first time we have determined the binding affinity and kinetics of the protein-protein interactions between EZH2 and p38 as well as with AKT1. The obtained results showed that EZH2 has higher binding affinity to p38 in comparison with AKT1, which will contribute to understanding of how EZH2 drives TNBC, and may provide new biomarkers of aggressive breast cancer, and targeted therapies. Our future studies towards mapping the interaction site, the potency and the nature of these PPIs, will give us insights as to whether these PPIs are “druggable” target and the feasibility of development of small molecules targeting the binding site of EZH2 to p38 and/or to AKT1.

#### **5. CHANGES/PROBLEMS**

No problems to report. No changes to the original aims and tasks.

#### **6. PRODUCTS**

None for this period

#### **7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS**

Zaneta Nikolovska-Coleska, Ph.D.  
Principal investigator (partnering PI)

**ORCID ID:**

Nearest person month worked: 1

Contribution to project: planned direction of project to follow SOW, designed experiments and guided the postdoctoral fellow, analyzed and summarizing the obtained results.

Lei Miao, Ph.D.  
Postdoctoral Fellow

**ORCID ID:**

Nearest person month worked: 3

Contribution to project: read the literature, designed experiments and discussed with PI, carried out experiments and analyzed the results.

#### **8. SPECIAL REPORTING REQUIREMENTS**

As this is a collaborative award, we will provide independent reports as required by DOD. This is the report of the Partnering PI, Dr. Zaneta Nikolovska-Coleska. The Initiating PI, Dr. Kleer, will provide a separate report.

#### **9. APPENDIX None**