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14. ABSTRACT:
This project focuses on the important but under-studied role of the P-TEFb-dependent transcription elongation machinery in human breast cancer progression. It aims to test the hypothesis that transcription elongation is a key regulatory step in breast cancer development, and that targeting P-TEFb can be an effective strategy to block breast cancer progression. During the current reporting period, we have established all the required biological assays and generated several key cell lines that either stably overexpress components of active P-TEFb complexes or knock down components of the inhibitory complex. These cell lines will be tested in various EMT, cancer stemness and invasion assays as originally proposed. We are also investigating the activity of a novel CDK9 inhibitor in blocking the expression of various transcription factors that control cell growth and EMT. By establishing these key reagents and experimental conditions, we are laying a solid foundation and making excellent progress toward achieving the stated goals of the project in the next reporting period.

15. SUBJECT TERMS

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1. INTRODUCTION:

Aberrant gene expression, caused by mutations in various signaling pathways, lie in the heart of breast cancer development and progression. Mammalian gene expression is controlled primarily at the level of transcription, which consists of several closely interlinked stages. During the past 30 years, the transcription field has been pre-occupied with the pre-initiation and initiation stages of transcription and ignored the subsequent elongation step, which in recent years has been shown to be extremely critical for the control of cell growth, embryonic development, as well as stem cell self-renewal and differentiation. This proposal focuses on the important but under-studied role of the transcription elongation machinery in human breast cancer progression and is designed to test the hypotheses that a network of P-TEFb-containing elongation complexes plays a key role in regulating breast cancer EMT, stemness, invasion and metastasis through controlling the expression of essential EMT and metastasis regulators, and that targeting P-TEFb is a viable therapeutic approach to halt breast cancer progression.

At the core of the elongation machinery is the CDK9 and cyclin T1 heterodimer termed P-TEFb that stimulates the transition of RNA Pol II from promoter-proximal pausing to productive elongation by phosphorylating Pol II and antagonizing negative elongation factors. In mammalian cells, P-TEFb is maintained in a functional equilibrium between the active and inactive states through reversible associations with distinct regulators that collectively form a network of P-TEFb complexes. Under normal growth conditions, more than half of nuclear P-TEFb are sequestered in a kinase-inactive complex called the 7SK snRNP that contains the 7SK snRNA as a structural scaffold, HEXIM1 as the kinase inhibitor, and LARP7 and MePCE as proteins that bind to and maintain the stability of 7SK snRNA. The 7SK snRNP represents the principle cellular reservoir of uncommitted P-TEFb and responds to demands for increased transcription and cell proliferation by releasing P-TEFb, which can subsequently be recruited by Brd4 to chromatin templates or integrated into the Super Elongation Complex (SEC) for transcriptional activation. The bromodomain protein Brd4 recruits P-TEFb to chromatin templates through interacting with acetylated histones and the mediator complex and is required for transcription of many primary response and signal-induced genes. In addition to P-TEFb, the SEC contains mostly fusion partners (e.g. AFF1, AFF4, ELL1, ELL2, ENL and AF9) of the mixed lineage leukemia (MLL) protein and promotes transcription of MLL-target genes, leading to some of the most severe forms of leukemia. Our working hypotheses is that P-TEFb activation as a result of shifting its functional equilibrium to the active side is a major driving force to promote breast cancer EMT, stemness and metastasis, and that the interference of this activation halts cancer progression and can thus be an effective therapy.

2. KEYWORDS:

Transcriptional elongation, P-TEFb, breast cancer, epithelial to mesenchymal transdifferentiation (EMT), invasion, metastasis

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Two specific aims have been proposed: 1) Determine whether the P-TEFb functional equilibrium can be perturbed to affect breast cancer EMT, invasion and metastasis, and whether a small molecule inhibitor of P-TEFb can be employed to halt breast cancer progression; 2) Determine why the EMT and metastasis-related genes are particularly sensitive to transcription elongation control and P-TEFb availability.

Research-Specific Tasks:

Major Task 1: Specific Aim 1: Determine whether the P-TEFb network can be manipulated to suppress breast cancer EMT, invasion and metastasis.	**Months**	**Researcher**
Subtask 1: Determine the roles of P-TEFb complexes in breast cancer EMT, stemness and metastasis in vivo. Overexpression or shRNA-based knockdown of various components of the P-TEFb complexes will be performed in breast cancer cells, and the effects of these manipulations on breast cancer EMT and metastasis will be determined in vitro and in vivo. Cell lines used: MCF10A progression model (MCF10A, M-II and M-IV cells), all in hand. Mouse strain used: 5-week old female nude mice (The Jackson Laboratory). 15 female mice per group (including controls) are needed in order to generate statistically significant results. For 14 experimental groups/cell line, with 2 cell lines, 420 female mice are needed.	1-30	X. Ji (Luo) H. Lu (Zhou)
Subtask 2: Determine whether small molecule CDK9 inhibitors can be used to halt breast cancer metastasis. 8 experimental groups to test various drug dosage and frequency regimes will be tested in vivo. An additional 8 experimental groups for tumor maintenance experiment and 3 groups for orthotopic experiment will be included. Cell lines used: M-IV cells and MB-MDA-231 breast cancer cells, all in hand. Mouse models used: Female nude mice and SCID mice (The Jackson Laboratory). 10 mice per group for 19 groups/cell line, with 2 cell lines: a total of 380 mice are needed.	7-36	X. Ji (Luo)
Milestone(s) Achieved: Generation of various stable cell lines with disrupted P-TEFb equilibrium. Determination of dosage and frequency of drug administration in vivo.	12-18	
Major Task 2: Specific Aim 2: Determine whether and why the EMT and metastasis-related genes are particularly sensitive to transcription elongation control and P-TEFb availability.	**Months**	**Researcher**
Subtask 1: Determine which SEC complex(es) mediates activation of EMT genes in breast cancer cells.	6-30	H. Lu (Zhou)

Cell lines used: MCF10A, M-II and M-IV.		
Subtask 2: Determine the molecular basis underlying high sensitivity of EMT and metastasis-related genes to control at the transcription elongation stage. Cell lines used: MCF10A, M-II and M-IV.	12-36	H. Lu (Zhou)
Milestone(s) Achieved: Detection of altered expression of one of more SEC components in human breast cancer; Detection of SEC binding to promoters of key EMT genes by ChIP; Successful ChIP-Seq analysis of P-TEFb components.	12-18	

What was accomplished under these goals?

Specific Aim 1: Determine whether the P-TEFb network can be manipulated to suppress breast cancer EMT, invasion and metastasis.

Subtask 1: Determine the roles of P-TEFb complexes in breast cancer EMT, stemness and metastasis in vivo.

We have established all the required biological assays and generated several MCF10A- and T47D-based cell lines that either stably overexpress components of active P-TEFb complexes or knock down the expression of components of inhibitory P-TEFb complexes. For example, we have successfully generated MCF10A cell lines stably expressing shRNA targeting LARP7 and HEXIM1. Figure 1 showed the stable knockdown of HEXIM1, a key 7SK snRNP component, in T47D cells and that this KD did not affect the proliferation of these cells.

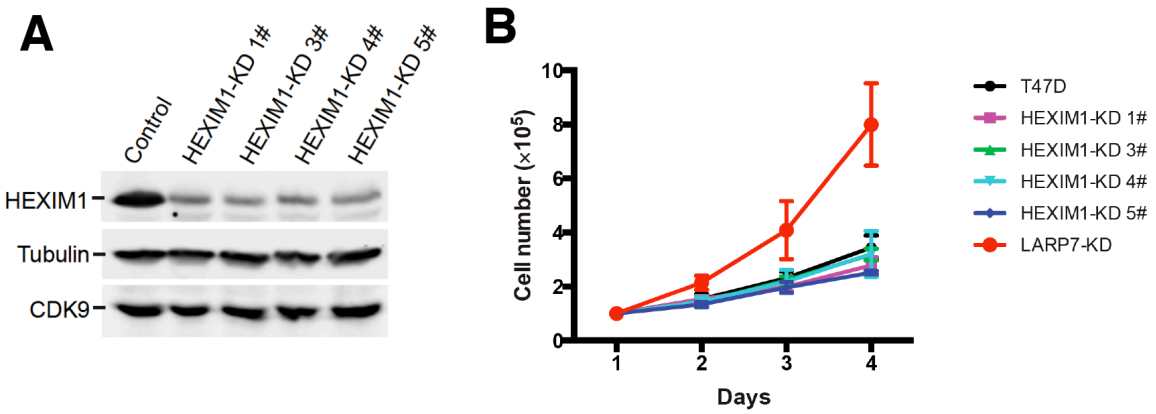

Fig 1. Knocking down HEXIM1 in breast cancer cells. A. *Western blotting confirmed the knockdown of HEXIM1 in T47D cells. Tubulin and CDK9 were used as loading controls. B. KD of HEXIM1 did not affect the proliferation of cells. LARP7 KD cells were used as a positive control for altered cell proliferation.*

We are in the process of establishing stable MCF10A- or T47D-based cell lines that overexpress SEC components AF9, AFF4 and ENL to determine whether this is sufficient to drive malignant progression. These cell lines will be tested in various EMT, cancer stemness and invasion assays as described in the research strategy.

Subtask 2: Determine the molecular basis underlying high sensitivity of EMT and metastasis-related genes to control at the transcription elongation stage.

We have also started to test the activity of a novel, highly selective CDK9 inhibitor, i-CDK9, in blocking the expression of various transcription factors that are critical for regulating breast epithelial cell growth and EMT. This inhibitor displays a 600-fold increased specificity for CDK9 than for other CDKs and acts synergistically with the Brd4 inhibitor to inhibit cancer cell growth and induce cell apoptosis (Figure 2).

Interestingly, we have recently shown that iCDK9 effectively blocked the expression of Myc and Slug genes in invasive T47D shLARP7 breast cancer cells. This confirms that the increased EMT and transformation found in LARP7 KD breast cancer cells are indeed due to the elevated P-TEFb activity.

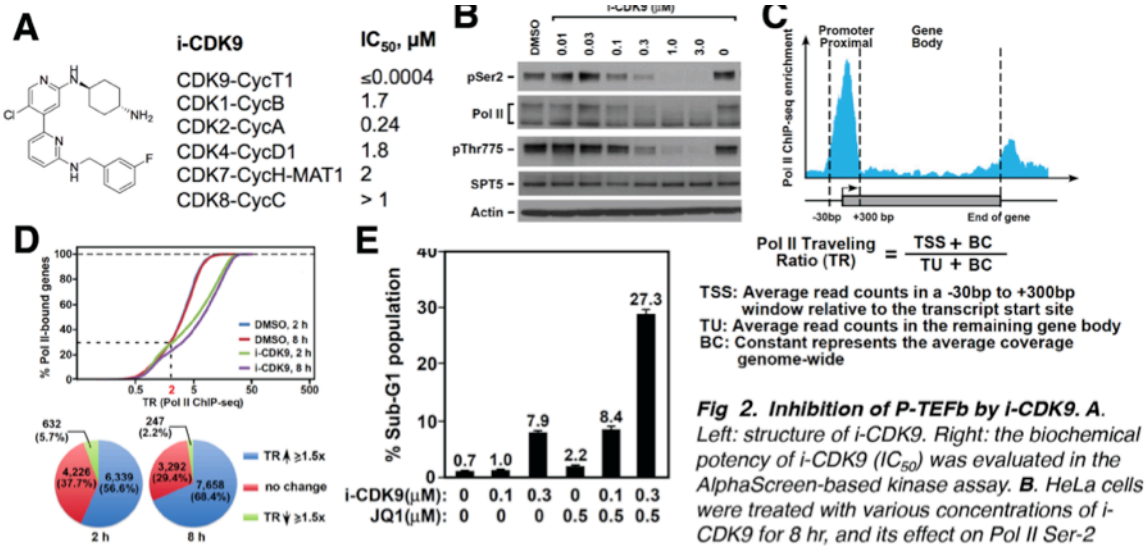

Fig 2. Inhibition of P-TEFb by i-CDK9. A. *Left: structure of i-CDK9. Right: the biochemical potency of i-CDK9 (IC_{50}) was evaluated in the AlphaScreen-based kinase assay.* ***B.*** *HeLa cells were treated with various concentrations of i-CDK9 for 8 hr, and its effect on Pol II Ser-2 phosphorylation was analyzed by Western blot.* ***C.*** *Schematic illustration of the Pol II traveling ratio (TR).* ***D.*** *CHIP-seq examined the distribution of Pol II-bound genes with a given TR under various conditions as indicated. The pie charts below described the fraction of genes with 1.5 fold increase, 1.5 fold decrease or no change in TR after exposure to i-CDK9 for 2h and 8h as compared to DMSO. E. Hela cells were treated with the indicated concentrations of i-CDK9 either alone or together with the Brd4 inhibitor JQ1, and apoptosis of the cells was measured by flow cytometry.*

Specific Aim 2: Determine whether and why the EMT and metastasis-related genes are particularly sensitive to transcription elongation control and P-TEFb availability.

Subtask 1: Determine which SEC complex(es) mediates activation of EMT genes in breast cancer cells.

We have started to test the importance of the SEC and Brd4-P-TEFb complexes in mediating the expression of key growth and EMT genes in breast cancer cells by knocking down key components of the SEC. Based on the structural studies of cyclin T1 and SEC component AFF4, we have recently identified a mutant cyclin T1 called AAG that no longer assembles into the complete SEC complex (Figure 3). This mutant cyclin T1 thus allows us to investigate the function of the SEC complex without affecting the Brd4 or PTEFb molecules.
We have employed the CRISPR/Cas9 genome-editing tool to knock out the gene encoding the SEC component AFF4 or knock in a mutant cyclin T1 (AAG) that blocks SEC assembly in the invasive T47D shLARP7 cells or MB-MDA-231 cells. Stable knockout or knockin cell lines have been obtained. Preliminary transformation and cell migration assays suggest that the SEC complex plays an essential role in allowing the activated P-TEFb

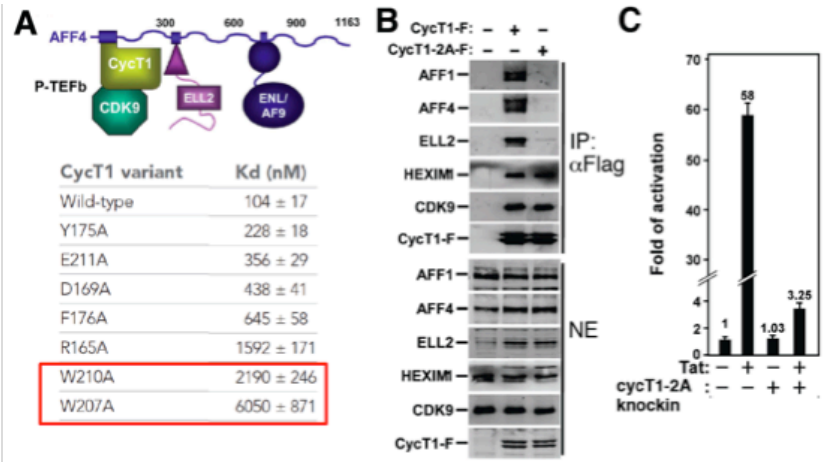

***Fig 3. Disruption of SEC assembly by CycT1-2A mutation. A**. Top: schematic drawing showing that direct binding of CycT1 to AFF4 allows the assembly of SEC to P-TEFb. Bottom: mutation of W210 and W207 to A greatly impaired binding of CycT1 to AFF4. **B**. Mutation of both W210 and W207 (CycT1-2A) disrupted assembly of SEC components to cycT1/CDK9. NE: nuclear extract. **C**.CycT1-2A mutant failed to support Tat transactivation in a luciferase reporter assay.*

to promote transformation, EMT and cell invasion. This confirms our hypothesis that the SEC mediates the activity of P-TEFb in promoting breast cancer EMT and invasion. We are in the process of testing the requirement of the Brd4-P-TEFb complex in these processes.

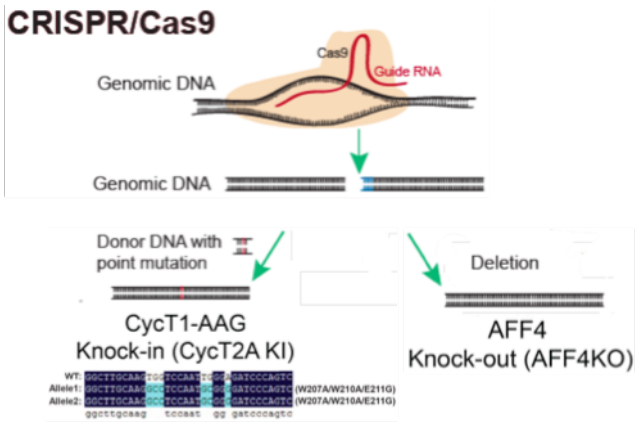

Fig 4. CRISPR/Cas9 strategy are used to introduce the Cyc T1–AAG mutant or abolish AFF4 expression

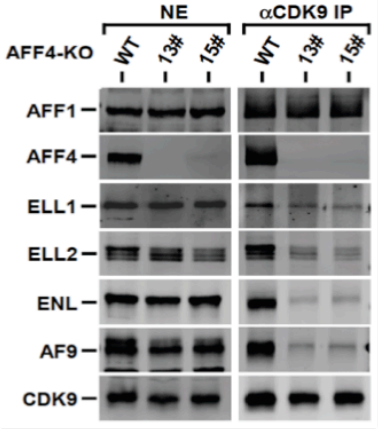

***Fig 5. Cyc T1–AAG mutant or AFF4 KD disrupt SEC assembly.** P-TEFb that is assembled into the SEC complex was isolated by anti-CDK9 IP from nuclear extracts (NE) and detected by western blotting with various antibodies. NE was loaded as controls.*

Subtask 2: Determine the molecular basis underlying high sensitivity of EMT and metastasis-related genes to control at the transcription elongation stage.

To be started in year 2.

In summary, by establishing the key reagents and experimental conditions as described above, we are laying a solid foundation and making excellent progress towards achieving the goals of the project, which will be continued in the next reporting period.

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

This project represents a major focus of research conducted in both the Zhou and Luo laboratories. It applies the concepts and experimental techniques derived from multiple disciplines and thus offer Dr. Xiao-dan Ji and Dr.

Tongge Zhu, two postdoctoral researchers in the Luo lab and Dr. Hengyi Shao, a new postdoctoral researcher jointly sponsored by the two laboratories, an excellent opportunity to become exposed to and familiar with the languages and tools used in the areas of biochemistry, molecular cell biology, and bioinformatics. Through supervising, training, coordinating, recruiting, motivating, writing and defining research directions for all specific aims, Dr. Zhou and Dr. Luo, the principal investigators of these two partnering awards have been intimately involved in every aspect of the project. In addition, the two PIs have taught beginning graduate students to set up experiments, and used the weekly joint lab meetings and journal clubs as opportunities to train the students and postdoctoral researchers to better organize their data and thoughts and give more succinct and impressive presentations.

The scientific environment at UC Berkeley, where this project is being performed, also provides excellent opportunities for intellectual growth and collaboration for the researchers associated with this project. Many regularly scheduled seminars encompassing all areas of modern biology are available and can benefit this project. The MCB Department and the Division of Biochemistry and Structural Biology and Division of Cell and Developmental Biology, to which the Zhou and Luo laboratory belongs respectively, organize annual retreats where graduate students and postdoctoral researchers from the two laboratories have opportunities to present their latest findings and obtain valuable feedbacks. Additional interactions are frequent between our two labs and those of Drs James Hurley, Britt Glaunsinger, Robert Tjian, Jennifer Doudna, Michael Botchan, and Michael Rapé, among others, and provide further intellectual support, technical help with experiments and useful reagents/tools. Moreover, many UC Berkeley labs are at the forefront of technology innovation, providing the researchers in the two labs with an opportunity to take advantage of the best new methods in proteomics, computational, imaging, genomic editing, and structural analyses. In summary, the breadth and depth of the UC Berkeley scientific environment where the Zhou and Luo laboratories are located provide unparalleled opportunities for training and professional development for all the researchers working on this project.

How were the results disseminated to communities of interest?

Nothing to Report

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

During the next reporting period, we will continue to determine the effects of disrupting P-TEFb functional equilibrium on breast caner progression and investigate the mechanism by which P-TEFb activation promotes breast cancer invasion and metastasis. Specifically, to accomplish the remaining goals in Aim 1, various in vitro and in vivo biological assays will be performed to evaluate the malignant statuses of various newly generated stable cell lines, in which the various subunits of the three major P-TEFb complexes are either overexpressed or knocked down. We will also characterize the effects of the highly selective CDK9 inhibitor, i-CDK9, either alone or in combination with the Brd4 inhibitors, on breast cancer progression in the assays. The Luo lab will take the lead in coordinating these experiments. For Aim 2, the requirements for both SEC and Brd4-P-TEFb complexes in breast cancer progression will be determined using the engineered cell lines generated this year and several Brd4 inhibitors we have obtained. In addition, we will identify direct P-TEFb-target genes among the EMT transcription factors and CSC stemness factors. Based on this information, genome-wide ChIP-seq assay will be performed to evaluate the pausing index for RNA Pol II. The potential role of a Super Enhancer will also be tested by knocking down the mediator complex in these breast cancer cells and test whether this blocks breast cancer progression. The Zhou lab will be largely responsible for these aspects of the study. Taken together, with the establishment of key reagents and experimental conditions, we are on track to test the hypotheses put forward in the proposal.

4. IMPACT:

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

The successful completion of the project will not only establish a new conceptual paradigm, but also have important clinical implications in halting breast cancer progression and metastasis. Specifically, our study will confirm the components of the general transcription elongation machinery as an important factor to drive the metastasis of breast cancer. By employing a novel highly selective P-TEFb inhibitor in the proposed experiments, we will also directly test the idea that targeting the P-TEFb network of complexes can be an effective strategy to treat metastatic breast cancer.

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.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report.

Changes that had a significant impact on expenditures

Nothing to Report.

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

Nothing to Report.

6. PRODUCTS:

Publications, conference papers, and presentations

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

New breast cancer stable cell lines have been established. They will be freely shared with the scientific community upon request.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Qiang Zhou
Project Role:	Initiating PI
Researcher Identifier:	qzhou (eCommon ID)
Nearest person month worked:	4
Contribution to Project:	Dr. Zhou supervises, trains, recruits, and motivates all personnel on the project. He also coordinates and defines research directions for all specific aims together with Dr. Kunxin Luo, the partnering PI
Funding Support:	This award

Name:	Hengyi Shao
Project Role:	Postdoctoral researcher
Researcher Identifier:	Hengyishao (eCommon ID)
Nearest person month worked:	6
Contribution to Project:	Dr. Shao has performed various EMT, cancer stemness and invasion assays to test whether activation of P-TEFb leads to EMT and metastasis of breast cancer cells
Funding Support:	This award

Name:	Huasong Lu
Project Role:	Graduate student
Researcher Identifier:	Luhuasong (eCommon ID)
Nearest person month worked:	1
Contribution to Project:	Mr. Lu has established the M-IV-based cell lines stably knocking out the SEC component AFF4 or expressing a mutant cyclin T1 called AAG that no longer assembles into the complete SEC complex
Funding Support:	NIH

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

None.

8. SPECIAL REPORTING REQUIREMENTS:

An independent report will be submitted by the partnering PI Dr. Kunxin Luo.

9. APPENDICES:

None