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TITLE: Epigenetic Targeting of the Cancer Stem Cell Hierarchy in Triple-Negative Breast Cancer

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14. ABSTRACT

We found EZH2 is required for CSC maintenance in TNBC lines. A comparison of EZH2 inhibitor and EZH2 knockdown cell gene expression shows EXH2 represses IL-10 a mediator of anti-tumor immunity. Thus EZH2 inhibitors may reactivate antitumor immune surveillance. EZH2 inhibition also releases EZH2 to serve as an activator of genes that drive NFKB action, providing a rationale for dual EZH2 and NFKB blockade.

We sorted ALDH1+ from ALDH1- cells. ALDH1+ cells have >82% purity and good viability in both MDA-MB-231 (231) and MDA-MB-468 (468). ALDH1+ population is enriched for sphere forming cells and expresses higher DOT1L, H3K79me2 and embryonic stem cell transcription factors Myc and Sox 2 and appears to be uniquely sensitive deletion of CSC by DOT1L inhibition. RNASeq and ChIP Seq assays in the next year should inform the gene programs directed by DOT1L required for its CSC maintenance role and for tumor initiating cell action in vivo.

15. SUBJECT TERMS
Triple Negative Breast Cancer, histone, cancer stem cells, epigenetics, histone methyl transferase inhibitor, DOT1L inhibitor, EZH2 inhibitor

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Year 4 NCE FINAL Report W81XWH-15-1-0581-
"Epigenetic Targeting of the Cancer Stem Cell Hierarchy in Triple-Negative Breast Cancer"

Summary of grant findings:

This grant investigated the role of EZH2 in triple negative breast cancer (TNBC) stem cell maintenance and the therapeutic potential of novel EZH2 inhibitor drugs for this deadly form of breast cancer. We had found EZH2 is required for CSC maintenance in TNBC lines. A comparison of EZH2 inhibitor over 7 days and EZH2 knockdown cell gene expression shows EZH2 represses IL6, IL8, IL1-beta, and IL-10 all drivers of the NFKB pathway. EZH2 inhibition appears to activate the NFKB pathway thereby upregulating PDL1 RNA and cell surface expression in 4 different TNBC cell lines. These data provide a clinical rational for testing the anti-tumor efficacy of combinations of EZH2inhibitors used together with either NFKB or checkpoint inhibitors.

We tested the potential role of a novel DOT1L inhibitor, EPZ5676 to target TNBC cancer stem cells (CSC). We sorted ALDH1+ from ALDH1- cells from both MDA-MB-231 (231) and MDA-MB-468 (468). We found the ALDH1+ population is enriched for sphere forming cells and expresses higher DOT1L, H3K79me2 and embryonic stem cell transcription factors Myc and Sox2 and appears to be uniquely sensitive deletion of CSC by DOT1L inhibition. RNASeq has now been completed and ChIP Seq for DOT1L and H3K79me3 will be completed in the next few months as will in vivo assays testing effects of DOT1L inhibitor to reduced TNBC CSC. This work will identify gene programs directed by DOT1L that are required for its CSC maintenance role and for tumor initiating cell action in vivo.

INTRODUCTION:

This grant aims to use epigenetic drugs to selectively deplete tumor initiating stem cells (T-ISC) within Triple Negative Breast Cancer (TNBC). Cancer stem cell (CSC) subsets generate progeny with reduced sphere formation, tumorigenicity and metastatic potential. CSC subpopulations from both dissociated TNBC tumor cultures and TNBC lines express an genes signatures associated with stem cells, and metastasis, and are highly chemotherapy and radiation resistant. Epigenetic mechanisms govern stemness and lineage commitment and histone methylation governs expression of the master transcription factors that maintain stem cell self-renewal. We showed the $CD44^{+}CD24^{low+}$ "precursor" subpopulation was killed by a DOT-like protein 1 (DOT1L) inhibitor and its $CD44^{+}CD24^{neg}$ "progeny" were selectively killed by an Enhancer of Zeste Homologue 2 (EZH2) inhibitor. The present grant further investigates the **hypothesis** is that DOT1L and EZH2 maintain subpopulations of TNBC tumor initiating stem cell (T-ISC) subpopulations and that these differences can be exploited therapeutically to effectively eliminate these T-ISC subsets using recently developed DOT1L and EZH2 inhibitors with or without chemotherapy. **AIM 1** will test *in vitro* effects of DOT1L and EZH2 on stem cell features, lineage commitment, proliferation and survival in CSC/ T-ISC subsets from TNBC lines and primary breast cancer. **AIM 2** ***i)*** will identify EZH2 and DOT1L target genes using global RNAseq and ChIPSeq to correlate gene expression and K3K27me2 and K3K79me2 patterns in sorted CSC subpopulations +/- DOT1L or EZH2 inhibitor drugs and ***ii)*** will assay effects of EZH2 inhibition on NFKB driven stem cell self-renewal and effects of DOT1L inhibition on Wnt/β-catenin-driven stem cell regulators. **AIM 3** will test effects of DOT1L and EZH2 inhibitors on tumor initiation frequency, tumor growth and metastasis in vivo and the potential for combinatorial synergies between these and chemotherapy in xenografts in vivo

KEYWORDS:
Triple Negative Breast Cancer, histone, cancer stem cells, epigenetics, histone methyl transferase inhibitor, DOT1L inhibitor, EZH2 inhibitor,

ACCOMPLISHMENTS:

1. What were the major goals of the project?

The major goals of the project remain as stated in the approved SOW. Study goals are listed Table 1 below. To date we have completed AIM1 and over half of AIM2 and will complete AIM2 and 3 in the NCE yr 4.

The Table below summarizes the goals and work completed in the first year.

TABLE 1 PROJECT GOALS		**ACCOMPLISHMENTS**
Specific Aim 1 Test the effects of DOT1L and EZH2 inhibition/knockdown on in vitro stem cell features, lineage commitment, proliferation and survival in CD24^{low+} and CD24^{neg} T-ISC subsets from TNBC lines and primary breast cancers	**Planned Timeline**	**Work completed (% completed)**
Major Task 1 Effects on stem cell phenotype, cell proliferation, and survival in vitro	Months	
Subtask 1 Dose titration in asynchronous and sorted cells in multiple cell lines with different drugs	1-6	100% completed
Subtask 2 Test drugs effects on In vitro stem cell phenotypes	1-6	100% completed in MDA-MB-231
Subtask 2 Test drugs effects on In vitro stem cell phenotypes		100% completed in 2nd line MDA-MB-468 for Dot1L inhibitor
Subtask 3 Test drug effects on Embryonic stem cell transcription factors (ES-TFs)	6-9	Completed for MDA-MB-231 AND MD-MB-468 (DOT1L inhibitor) (100% complete)
Milestone(s) Achieved 1.Identify lowest drug doses that affects stem cell self-renewal without affecting viability or cell cycle 2.Define if drugs alter in vitro stem cell properties and self renewal and 3. Determine if ES-TFs expression is modified by DOT1L or EZH2 inhibitors or knockdown	1-9	In yr 1 we identified LD 50 of drugs for 4 lines. In yrs 2, drug titrations show H3K27Me3 loss with EZH2 inhibitor and H3K79Me2 inhibition by Dot1L inhibitor EPZ5676. Cell cycle is not affected by drug doses that inhibit CSC (spheres, % ALDH1+, ES-TFs) in vitro. Yr3 we confirmed these finding for both drugs in 2nd TNBC lines. 100% complete. In yr 4 NCE, **in vivo xenograft assays AIM 3 now shows DOT1L inhibitor targets specifically the TNBC CSC population and decreases tumor initiating cells.**

Local IRB/IACUC Approval for work of AIM 3	3	Approval requested
Milestone Achieved: IACUC Approval	6	Approval obtained
Major Task 2 Effects on lineage commitment		
Subtask 1 transduce a red fluorescence protein (RFP)-tagged ShEZH2 lenteviral vector(ref) into sorted $CD24^{neg}$ cells from MDA-MB-231	1-24 mo	EZH2 and DOT1L ShRNA stable lines selected in MDA-MB-231
Subtask 2 Test if ShEZH2 affects ability of CD24neg cells to generate spheres and their phenotype	1-24 mo	EZH2shRNA effects on Spheres, ES-TFs and ALDH1+ is being assayed. 100% complete
Subtask 3 Test if isolated GFP+ CD24low cells contribute to sphere formation after DOT1L inhibition	1-24 mo	Near completion. Showed DOT1L inhibitor decreases Spheres, %ALDH1+ and ESTF expression. Validating findings with ShDOT1L
Milestone(s) Achieved: will have shown if EZH2 is required for CSC self renewal and if DOT1L is needed for sphere formation		100% completed in yr 4 of grant
Specific Aim 2 Test if EZH2 activates ES-TFs to drive self-renewal, and compare global gene expression in CSC TNBC subsets +/- EZH2 inhibitor and +/-shEZH2		*100% complete RNA Sequencing completed for 231 cells +/- EZH2 inhibition and knockdown and shows cooperation of EZH2 with NFKB pathways*
Subtask 3: *To test if EZH2 coactivates NFκB targets to drive $CD24^{neg}$ T-ISC self-renewal, ChIP assays will test if EZH2 binds to promoters of NFKB driven cytokine genes such as IL-6 that drive CSC self renewal.*	*24-36*	*RNA Seq of EZH2 inhibitor vs shRNA EZH2 showed that EZH2 inhibitor releases EZH2 to activate NFKB driver genes that in turn activate PDL1 expression on tumor cells and decrease anti-tumor immune surveillance. This is a pathway of drug resistance. We are currently testing if combined EZH2 and Checkpoint inhibitors in vivo improve drug response in immunocompetent mice to improve antitumor action.*
Subtask 4 *Since EZH2 appears to activate NFκB*	*36-48*	*Plan unchanged*

target genes to drive T-ISC self-renewal, inhibitors of NFKB might cooperate with EZH2 inhibitors to decreased expression of NFKB target genes		*Final In vivo work started and will be complete by end of May 2020.*
Milestone(s): will have shown EZH2 drives stemness by activating the NFKB pathways. This lays the foundation for using combined NFKB and EZH2 inhibitors together (See work proposed in NCE)		*95% completed see above.*

2. *What was accomplished under these goals?*
***Italics indicate new work accomplished in year* 3.**

ALDH1 flow sorting: *We sorted ALDH1+ from ALDH1- cells and using flow cytometry. Cells have >82% viability and sufficient numbers for assays required in both MDA-MB-231 (231) and MDA-MB-468 (468) (Fig 1A). We showed the ALDH1+ population is enriched for sphere forming cells and expresses higher DOT1L, H3K79me2 and embryonic stem cell transcription factors Myc and Sox 2 (Fig 2B)*

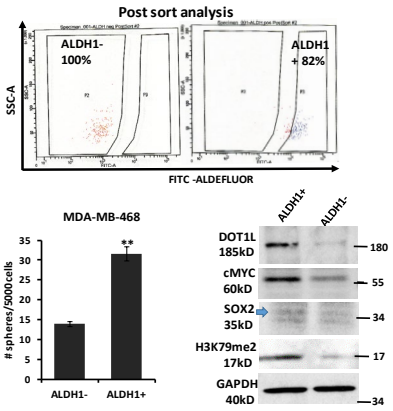

Fig 1 A. Enrichment of ALDH1+ cells from ALDH1- by flow sorting in MDA-MB-468. B. ALDH1+ cells show higher sphere formation (left) and higher DOT1L, H3K79Me2 and ES-TF expression (right).

Major Task 1 Effects on stem cell phenotype, cell proliferation, and survival in vitro

Subtask 1 Dose titration in asynchronous and sorted cells in multiple cell lines with 4 different drugs

Cell growth Inhibition (CTG) assay

Dose titration of two EZH2 inhibitors (GSK126 and EPZ5687) and of two DOT1L inhibitors (SGC0946 and EPZ5676) was reported in three TNBC cell lines: MDA-MB-231, SUM149 and SUM 159 and one leukemic cell line MOLM 13 using the Cell Growth Inhibition (CTG) assay from CellTiter-Glo.

Drug effects on Histone H3 Methylation

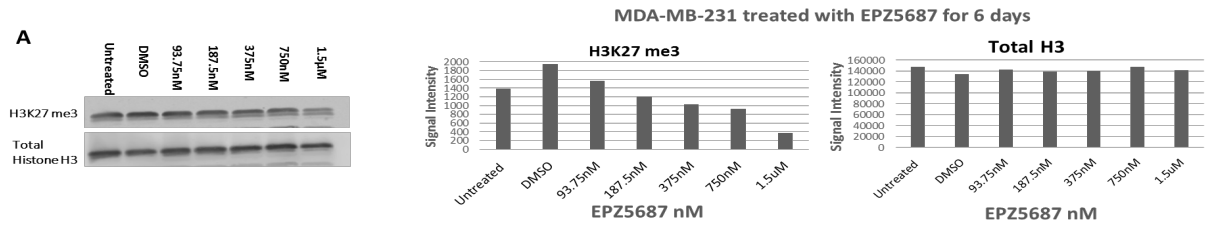

Figure 2 Western show the EZH2 inhibitor, EPZ5687, decreases H3K27me3 MDA-MB 231 after 6 days. Bar graphs represent the densitometric quantification of the Westerns. Total H3 levels were unchanged

Both EZH1 and Dot1L inhibitors have been titrated to show inhibition of their Histone H3 lysine methylation targets See Fig 2 and 3. Histones were extracted from MDA-MB-231 unsorted cells and total and H3K27me3 and H3 K79 me2 detected by blotting. We showed that EZP5687, the EZH2 inhibitor, decreases Histone H3K27me3 at doses of 750nM (Fig 2) and 100- 300nM of DOT1L inhibitor, EPZ5676 decreases H3K79Me2 in MDA-MB231 (Fig 3*). We have now completed dose titration of a second line MDA-MB-468 for EPZ5676 and completed in vivo*

studies of drug action on ALDH1+ enriched stem cells in a limiting dilution assay. This work is nearly complete and indicates that DOT1L inhibition targets stem cells and reduces the T-ISC in vivo.

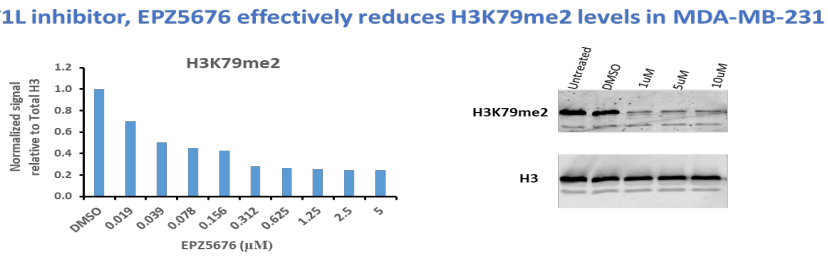

Figure. 3. Effect of DOT1L inhibitor EPZ5676 on H3K79me2 levels in MDA-MB-231 Western shows loss of H3K79Me2 in MDA-MB-231 cells treated with DOT1L inhibitor EPZ5676. Bar graph represents the quantified and normalized H3K79me2 signal intensity relative to total H3 levels at increasing concentrations of the drug. Max inhibition of H3K79ME2 was seen between 300-600nM drug.

Effects of DOT1L inhibition on cell number/time and viability

Cells were treated with DOT1L inhibitor EPZ5676 1μM over 10 days. DOT1L inhibition did not decrease proliferation over 7-8 days of drug exposure at most doses tested (Fig 4A) and had no effect on cell viability as measured as absorbance emitted on MTT dye reduction by live cells (Fig 4). DOT1L inhibitor did not change cell cycle distribution over 7 d in culture (Fig 4C). At doses of 1-5 μM in 231 and 0.25-1 μM in 468, DOT1L inhibition does not arrest cell cycle, decrease population growth or cell viability. Thus, any effects observed on % sphere formation or ALDH1 at this does cannot be due to growth inhibition or death of the bulk population.

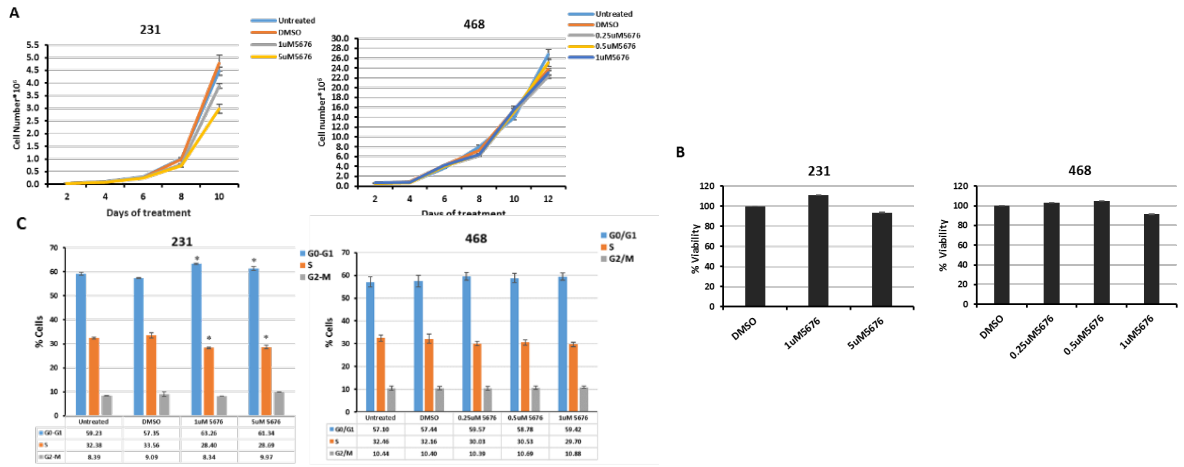

Figure 4. DOT1L inhibitor does not affect A) cell number over 10 days; B) cell viability or cell cycle profiles in MDA-MB-231 and in MDA-MB-468.

Similar assays were carried out with EZH2 inhibitors EPZ5687 and GSK126. Both drugs did not affect cell number increase over time, cell viability or cell cycle distribution.

Drug effects on Stem cell phenotype

Having defined the IC50 doses and shown that these drugs did not affect cell cycle or cell viability in our TNBC lines, we went on to test effects on stem cell markers, spheres and ALDH1% of DOT1L and EZH2 inhibitor doses in unsorted MDA-MB-231 and in Sum149. *We*

found the DOT1L inhibitor decreased %ALDH1+, spheres and ES-TFs in both the MDA-MB-231 cell line and a second TNBC line, MDA-MB-468 line (see Fig 5). Notably doses of 1-5 uM of

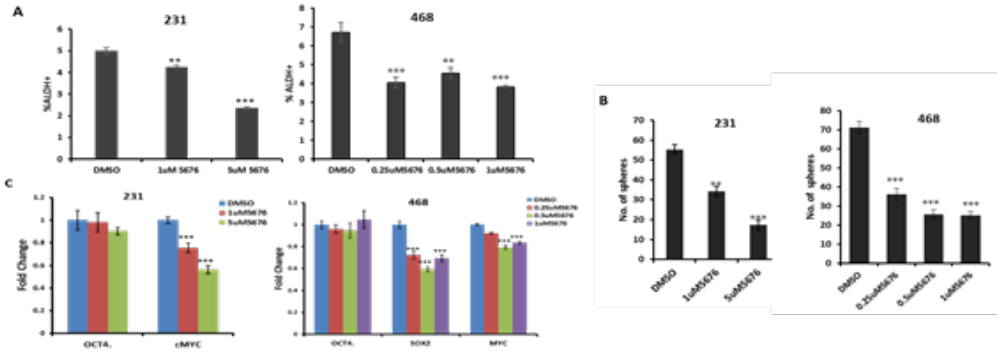

Fig 5. DOT1L inhibitor, EPZ5676 significantly decreased TNBC stem cell-like features A. % ALDH1+ cells analyzed using ALDEFLUOR assay. B. Mammosphere forming ability. C. Levels of ES-transcription factors: OCT 4 and cMYC in 231 and OCT4 cMYC and SOX2 in MDA-MB-468.

the *EZH2 inhibitor EPZ5687 also decreased sphere forming cells and ALDH1+ cells in MDA-MB-231 and Sum149 (not shown).*

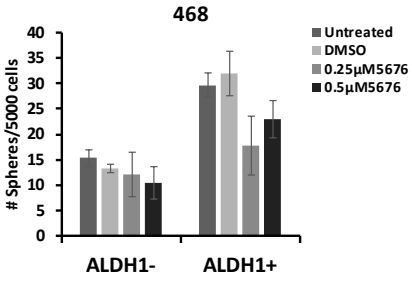

Figure6. DOT1L inhibitor EPZ5676 decreases sphere formation in ALDH1+ cells but not in ALDH1- populations.

With our new method for sorting ALDH1+ and ALDH1 negative stem cell subsets, we will now complete assays of drug effects on 1) Flow cytometry showed drug does not affect cell cycle in either population- thus cells are cycling when plated into sphere assays. 2) Sphere formation assays (Fig 5B); 3) CSC markers were decreased in the ALDH1+ stem cell enriched population but not in ALDH1- cells. Thus, only ALDH1+ cells from both 231 and 468 liners are sensitive to DO1L inhibition, while ALDH1- cells are resistant to effects of the DOT1L inhibitor See Figure 6.

EZH2shRNA causes a loss of sphere formation and decreases the % ALDH1+ cells

Two independent MDA-MB-231 sub-lines with ShRNA EZH2 have been established. Loss of EZH2 expression reduces the abundance of sphere forming cells and % ALDH1 cells but does not affect viability or proliferation rate (not shown).

We have completed RNA Seq of 231 cells +/- EZH2 inhibition with EPZ5687 and +/- ShEZH2. This year a new DOD grant funded PDF joined the lab and we were able to confirm that EZH2 inhibition with both EPZ5687 and EPZ0011989 both lead to activation of NFKB, and NFKB-dependent induction of PDL1 gene and cell surface PDL1 expression. Thus, a critical mechanism of resistance to EZH2 inhibitors could arise through activation of the inflammatory NFKB pathway in drug treated tumors, leading to induction and cell surface expression of greater PDL1 expression. Increased expression of PDL1 might lead in vivo to failure of ESZH2 inhibitor monotherapy but might lead to greater potential responsiveness to checkpoint inhibitor. In our grant final year, we confirmed the upregulation of PDL1 by two different EZH2 inhibitor drugs in 3 different TNBC lines. Syngeneic tumor models are now being used to test in vivo if combined checkpoint inhibitor and EZH2 inhibitor therapy show synergistic effects to inhibit TNBC growth in vivo. This work will be completed by May 31.

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

This project supported the work of one technician, a graduate Student, Hetakshi Kurani who is now completing her PhD work on the DOT1L part of the grant. It also supported a post-doctoral fellow, Myoung Shin in year 2, 3. In late year 3, and the NCE year, another PDF , Dr Harikumar Kuzhuvelil joined the lab to work on the ESZH2 inhibitor experiments. Trainees meet weekly or bi-weekly for one on one mentoring sessions with the PI and we meet as a project team all together every 2-3 weeks. These trainees participate in lab meeting weekly where we present our research progress and get critiques in a supportive friendly environment for improvement of research plans and presentation skills. Trainees also have numerous opportunities to attend seminars from UM researchers and invited speakers from each of 4 different molecular biology graduate program seminar series and the Cancer Center's Distinguished lecture series. Individuals with advanced professional skills and experience assist others in attaining greater proficiency. I also encourage all of my grant funded participants to attend scientific conferences particularly the Miami Winter symposium, a local high quality international meeting, and to attend the Cancer Center's international Epigenetics Symposia that are held regularly. Dr Shin presented a poster at the Miami Winter Symposium in Jan 2018.

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

Manuscripts reporting our findings on the targeting of CSC by both DOT1L inhibitor and EZH2 inhibitors are being written up. The EZH2 inhibitor project will report the synergy between EZH2 and Checkpoint inhibitor therapy for TNBC in immune competent mouse tumor models.

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

The goals remaining to be accomplished are summarized in the table below

GOALS TO BE ACCOMPLISHED in NCE		**TIME FRAME**
Specific Aim 2 Test if DOT1L activates ES-TFs to drive self-renewal in ALDH1+ vs ALDH1- cells, and compare global gene expression and H3K27me3, H3K79me2 methylation patterns in TNBC CSC subsets +/- drug inhibitors	24-36 mo	*RNA Seq was completed for EZH2 treated and control cells. RNA Seq and ChIP Seq are near completion for ALDH1+ and ALDH1- sorted cells with and without Dot1L inhibitor.*
Major Task 1 Test if i) EZH2 activates NFKβ to mediate self-renewal and ii) if DOT1L activates Wnt pathway targets		
Subtask 1 To test if *i) EZH2 activates NFK□□to mediate self-renewal and* ii) ifDOT1L is coactivating different transcription factors to target genes, we will using ChIP assays to test if Dot1L binds to promoters of Wnt target genes such as β catenin in flow sorted ALDH1+ cells but not ALDH1- cells	36-48 mo	Plan unchanged *i) We showed using RNA Seq of 231 +/- drug inhibitor and +/- shRNA EZH2 that EZH2 activates NFKβ to mediate self-renewal in year 3.* ii) In the NCE, we completed RNA Seq +/- DOT1L inhibitor EPZ5676 and assay effects on Myc and

		other CSC regulating genes.100% completed in NCE
Subtask 2 Test if Dot1L binds β catenin gene promoter at TCF4 (Wnt-dependent) binding sites. Chip/reChIP will test if promoter sites are co-occupied by both DOT1L and TCF4. We will also test if Wnt inhibitors cooperate with Dot1L inhibitors to downregulate Wnt target genes such as β catenin	36-48 mo	Plan unchanged Analysis underway on data acquired in the completed in NCE
Major Task 2 Compare global gene expression, and H3K27me3 and H3K79me2 methylation in stem cell TNBC subsets with and without drug inhibitors	36-48 mo	Plan unchanged
Subtask 1 establish ChIP assays for H3K27me3 and H3K79me2 methylation	36-48 mo	Plan unchanged yr 4
Subtask 2 Prepare and purify DNA for ChiPSeq and RNA for RNA Seq from TNBC breast lines +/-drug	36-48 mo	Plan unchanged yr 4
Subtask 3 RNA Seq and Chip Seq assays and then data analysis of RNA Seq and Chip Seq	24-36 mo	*RNA Seq for cells treated with EZH2 inhibitor or ShRNA EZH2 vs controls was completed in yr 3*
Milestone(s): Correlate global H3K79me2 marking with H3K27me3, H3K4me3 profiles and gene expression Compare bivalent marked genes that lose bivalency during $CD24^{low+}$ to $CD24^{neg}$ transition with those that change during the transition from normal mammary stem cell (MaSC) to luminal progenitors; Define histone methylation status of embryonic stem cell transcription factors that are differentially expressed in TNBC T-ISC subsets.		RNA Seq and ChIP Seq for cells treated with Dot1L inhibitor or ShDOT1L vs controls was done in NCE YR 4
Specific Aim 3 Test effects of DOT1L and EZH2 inhibitors on tumor initiation frequency, tumor growth and metastasis in vivo and the potential for synergies between these and chemotherapy	36-48 mo	
Major Task 1 ***Drug effects on tumor initiating stem cell (T-ISC) numbers in TNBC in vivo models***	36-48 mo	Complete in yr 4
Subtask 1 Will test if DOT1L inhibitor treatment for 7 days prior to injection decreases abundance of T-ISC in $CD24^{low+}$ cells as assayed by number of tumors formed using limiting dilution tumor formation assays of treated and untreated cells injected into MFP of host mice	36-48 mo	Complete in yr 4
Subtask 2 Will test if EZH2 inhibitor alone, NFKB inhibtor alone or both together over 7 days prior to injection decreases abundance of T-ISC in TNBC as assayed by number of tumors formed using limiting dilution tumor formation assays of treated and	36-48 mo	Currently testing if Checkpoint and EZH2 inhibitors synergize to inhibit CSC

untreated cells injected into MFP of host mice		
Milestone(s) Achieved: Will identify if CD24 low TISC frequency is reduced by DOT1L inhibitor and if EZH2 inhibition abrogates TISC action in CD24neg cells		
Major Task 2 ***Test drug effects on growth and formation of metastasis from primary***	36-48 mo	In vivo work underway
Subtask 1 When DT28 $CD24^{low}$-derived tumors from 100,000 cell injections reach 1 cm, they will be excised and animals followed for metastasis. Effects of DOT1L inhibitor pretreatment on tumor growth rate and metastasis will be compared with untreated controls	36-48 mo	In vivo work underway
Subtask 1 When $CD24^{neg}$-derived tumors from the 100,000 cell injections reach 1 cm they will be excised and animals followed for development of metastasis. Effects of EZH2 inhibitor pretreatment on rate of tumor growth and mets formed will be compared with untreated controls	36-48 mo	In vivo work underway
Milestone(s) Achieved: Will demonstrate if DOT1L or EZH2 inhibitors delay growth of DT28 breast cancer xenografts and if either can prevent formation of metastasis from tumors formed by different T-ISC subsets		
Major Task 3 ***Test antitumor effects of DOT1L or EZH2 inhibitors and if either or both complement taxane therapy*****.**	24-36	In vivo work underway
Subtask 1 we will compare tumors formed in untreated controls, vs treatment with EZH2 inhibitor, or DOT1L inhibitor each for 7 days decrease tumors formed from 2 X 10^6 cells injected orthotopically in NSG mice	24-36	In vivo work underway
Subtask 2 to test if EZH2 or DOT1L inhibitors can cooperate with paclitaxel chemo, NSG mice will be injected with cells pretreated with either DOT1L inhibitor, EZH2 inhibitor as in Subtask 1 above. When tumors reach 1 cm, mice will be treated with weekly IV paclitaxel, tumor growth tested by IVIS and compared to tumors in the Subtask 1 that did not receive chemo.	24-36	In vivo work underway
Milestone(s) Achieved: Will show if DOT1L or EZH2 inhibitors impair TNBC xenograft growth or delay metastasis. Will also show if these drugs cooperate with paclitaxel to inhibit tumor growth		In vivo work underway

IMPACT:

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

i)We have shown that EZH2 plays a non-canonical role to induce expression of a driver of the NFKB pathway. This data formed the rationale for testing potential drug synergy between checkpoint inhibitors together with EZH2 inhibitors. This work is near completion and will be finished within the next 2-3 months (manuscript to be submitted).

ii) We made the novel observation that DOT1L appears to govern triple negative breast cancer (TNBC) stem cells. This may open avenues for treatment of this deadly disease with a DOT1L inhibitor, EPZ5676, that is well tolerated in humans and has been used in clinical trials for MDS and AML in humans. This would be the first application of this type of drug for TNBC. Our ChIP Seq and RNA Seq experiments may reveal additional targetable pathways regulated by DO1K in TNBC

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report yet. We are collaborating with Epizyme who have provided their lead compounds currently in clinical trials in humans with lymphoma/acute leukemia for this work. Our work may identify novel utility of these leukemia drugs for TNBC. We may also illuminate non-canonical roles for EZH2 that are revealed by comparison of EZH2 inhibitors and EZH2 knockdown.

What was the impact on society beyond science and technology?

Nothing to report yet.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report -- No changes to plan. We gave priority to testing effects of DOT1 L inhibitors on ALDH1+ sorted cells since the ALDH1- cells appear insensitive to this drug in vitro. We also tested combined ESH2 inhibitor EPX5687 effects + /- checkpoint inhibitors in unsorted cells rather than sorted, since this combination is supported by in vitro data.

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

Our first Specific Aim 1 was to test the effects of DOT1L and EZH2 inhibitors or knockdown on in vitro stem cell features, the emergence of CD24 negative cells from CD24 parental cells and how these drugs affected proliferation and survival in CD24low+ and CD24neg T-ISC subsets from TNBC lines and primary breast cancers. This work required the ability to isolate by the two stem cell sub-populations. This work was delayed in year 1 by the early departure of a Post-doc and late hiring of a new Post-doc (Dr Miyoung Kim) and also by difficulties we have had with sorting two populations of cancer stem cells needed for the purpose of this study (as described in the progress report above). In year 2, the lab technician working on the project left the lab in August 2017. We did not hire a replacement but rathe planned to and are now requesting a no-cost extension for this work as it will likely require longer to carry out remaining in vitro work of AIM 2 to support in vivo work of AIM3 and we want to carry this our over a NCE year.

Personnel Issues:

Work in year 1 was delayed by the departure of a seasoned Post-doc in Oct 2015. Postdoctoral fellow, Dr Diana Azzam whose PhD work formed the basis for this project, decided after one month on the project (Sept 1-Oct 1, 2015) to focus on a different project and left to take up a faculty position at a different institution. A Masters level technician Ms Joanna Poprawski, took

the project lead in Sept 2015 and was joined by a Grad Student Ms Hetakshi Kurani in March, 2016. A post-doc, Dr Myoung Shin joined the lab in September 2016. She completed the RNA Seq work for cells treated with EZH2 inhibitor and for cells with stable EZH2 knockdwon. Unfortunately she has also left the lab in Sept 2018. Dr Kuzhuvelil, a new PDF joined us in May 2019 and will complete the work of AIM 3.

Changes that had a significant impact on expenditures
The delay in hiring our post doc and grad student account for incomplete expenditure of funds allocated in the first year. Our technician left the team in Sept 2017. We will continue with PDF and PhD student and aim for an NCE to complete work in a 4rth yr.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report

Significant changes in use or care of human subjects
Nothing to report

Significant changes in use or care of vertebrate animals
Nothing to report

Significant changes in use of biohazards and/or select agents
Nothing to report

PRODUCTS:

Publications, conference papers, and presentations
Poster Presentation at Miami Winter Symposium, Miami, FL USA:
Title: Identification of Non-catalytic Targets of EZH2 in Triple-Negative Breast Cancer
Presented by: Miyoung Shin, Hyunho Yoon, Deukwoo Kwon, Yuguang Ban, Zhen Gao, Steven Xi Chen, Joyce M. Slingerland
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Journal publications
Nothing to report so far
Two manuscripts are being prepared and will be submitted by the end of the summer on the DOT1L and EZH2 inhibitor projects respectively.

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
New techniques will be reported at publication- no publications yet.

Inventions, patent applications, and/or licenses

Nothing to report

Other Products
Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

Dr. Evadnie Rampersaud is no longer at the institution. Dr. Steven Xi Chen, Director of the Biostatistic and Bioinformatics Shared Resource at Sylvester Comprehensive Cancer Center will serve as an unpaid scientific collaborator to design and oversee bioinformatics analysis.

What individuals have worked on the project?

Name:	Joyce M. Slingerland, MD, PhD
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0003-1487-8554
Nearest person month worked:	2.4
Contribution to Project:	Dr. Slingerland coordinated and supervised all experiments.
Funding Support:	Other funding source includes BCRF, FBCF, NIH-NCI
Name:	Claes Wahlestedt, MD, PhD
Project Role:	Collaborator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	N/A
Contribution to Project:	Assist with design and execution of dose titrations and assays of synergies between agents
Funding Support:	Other funding support from NIH- NINDS and NIH-NIDA
Name:	Ramin Shiekhattar, PhD
Project Role:	Collaborator
Researcher Identifier	N/A

(e.g. ORCID ID):	
Nearest person month worked:	N/A
Contribution to Project:	Will provide expertise for genome-wide analysis of CHipSeq/RNA Seq analysis of histone methylation and methyl transferase occupancy of the genome in AIM2
Funding Support:	Other funding support from NIH-NIGMS
Name:	Joanna Poprawski, MS
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	Was no longer working on this grant during year 3
Contribution to Project:	Plan and execute experiments. Gather and analyze data.
Funding Support:	100% from this grant
Name:	Miyoung Shin PhD
Project Role:	Post-doctoral Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	12
Contribution to Project:	Plan and execute experiments. Gather and analyze data.
Funding Support:	100% from this grant
Name:	Hetakshi Kurani, MS
Project Role:	Grad student
Researcher Identifier (e.g. ORCID ID):	N/A

Nearest person month worked:	12
Contribution to Project:	Plan and execute experiments. Gather and analyze data.
Funding Support:	100% from this grant
Name:	Harikumar Kuzhuvelil PhD
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	11 months worked full time on work of this grant
Contribution to Project:	Plan and execute experiments. Gather and analyze data
Funding Support:	Partly from this grant and partly from other sources.

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

Joyce Slingerland (PI):
New grants during the entire grant period

DOD BCRP Breakthrough Award W81XWH-17-1-00456 **2017-2020**
Breast Cancer Research Program – US Army **$250,750/yr**
Novel mechanisms whereby p27 drives tumor progression in PI3K-activated cancers
(PI)

NIH-NCI 1 R01 CA210440-01A1 **2017-2022**
Mechanistic links between changing estrogen profiles, inflammation **$221,887/yr**
and the increased risk and metastasis of breast cancer in obese women
(PI)

NIH-NCI 1 R01 CA210440-01A1 SUPPLEMENT **2018-2022**
NIH-NCI **$120,000 (total)**
Effects of Estrogen Profiles and Obesity on ER Positive Breast Cancer PDX Generation
(PI)

K12 CA226330-01 **2018-2023**
UM Calabresi Clinical Oncology Research Career Development Award
Slingerland (PI)

DOD Breakthrough Award W81XWH1910255 **2019-2022**
Breast Cancer Research Program – US Army **$250,189/yr**
Role of LBH in the Etiology of Basal-Subtype Triple-Negative Breast Cancer
(PI K Briegel, Co-PI Slingerland)

DOD Breakthrough Award W81XWH1910393 **2019-2022**
Breast Cancer Research Program – US Army **$20,000/yr**
Role of FANCA in Breast Tumorigenesis and Cancer Development
(PI, Y Zhang; Partnering PI, Slingerland)

What other organizations were involved as partners?
None