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Kinase-Mediated Regulation of 40S Ribosome Assembly in Human Breast Cancer

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CONTRACTING ORGANIZATION:
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14. ABSTRACT

The BCRP Breakthrough DOD_W81XWH-15-BCRP-BREAKTHROUGH-FL12) studies of the laboratories of Drs. Katrin Karbstein (Initiating Principal Investigator [PI], Scripps) and John Cleveland (Collaborating/Partnering PI, Moffitt Cancer Center) seek to validate 40S ribosome assembly as a therapeutic target for triple negative breast cancer (TNBC). Specifically, we have shown that the serine/threonine casein kinase-1δ (CK1δ) phosphorylates the 40S ribosome assembly factor Ltv1, and that selective knockdown or silencing of CK1δ, or forced expression of Ltv1 mutant that cannot be phosphorylated by CK1δ, blocks ribosome assembly and compromises the growth and survival of TNBC. Further, we have shown that forced overexpression of a phospho-mimetic Ltv1 mutant (i.e., constitutively active Ltv1) can override the deleterious effects of CK1δ□inhibitors or CK1δ silencing on ribosome assembly. Using genetic approaches, we will define roles of the CK1δ-to-Ltv1 circuit in TNBC growth and survival, and will assess if TNBC regression triggered by CK1δ inhibition or loss disables in ribosome assembly via effects on Ltv1. We have also developed TNBC tumor cells that are resistant to CK1δ inhibitors, and will assess if this resistance involves gain-of-function mutations in Ltv1, and if resistance can be overcome with drugs that direct destruction of ribosome assembly intermediates via the autophagy pathway.

15. SUBJECT TERMS

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ANNUAL REPORT: DOD_W81XWH-15-BCRP-BREAKTHROUGH-FL12

1. INTRODUCTION

The first-in-kind DOD-funded Breakthrough collaborative studies of the laboratories of Dr. Katrin Karbstein (Initiating Principal Investigator [PI], Scripps-Florida) and Dr. John L. Cleveland (Collaborating/Partnering PI, Moffitt Cancer Center) seeks to validate targeting 40S ribosome assembly as a therapeutic target for aggressive breast cancer subtypes, including triple negative breast cancer (TNBC) that currently lacks targeted therapies. Specifically our research team has shown that the serine/threonine casein kinase-1δ (CK1δ) phosphorylates the 40S ribosome assembly factor Ltv1 in both yeast and TNBC cells, and that selective knockdown or silencing of CK1δ, or forced expression of Ltv1 mutant that cannot be phosphorylated by CK1δ, blocks ribosome assembly in yeast and compromises the growth and survival of TNBC cells. Further, we have shown that forced overexpression of a phosphor-mimetic Ltv1 mutant (i.e., a constitutively active mutant) can override the deleterious effects of CK1δ inhibition or silencing on ribosome assembly. Thus, these DOD funded studies suggest that the CK1δ-to-Ltv1 circuit is a tractable vulnerability for TNBC and for other breast cancers that we have shown overexpress CK1δ such as HER2+ and luminal B breast cancer subtypes. Accordingly our DOD supported studies seek to validate this circuit as a therapeutic target for TNBC so these findings can be advanced into the breast oncology clinic. Our studies include those that seek to further understand the regulation and role of this circuit in TNBC breast cancer growth and survival, and to assess if TNBC regression that is triggered by treatment with CK1δ inhibitors involves defects in ribosome assembly. We will also test if TNBC tumors can develop resistance to CK1δ inhibitors, if such resistance involves the acquisition of gain-of-function phosphomimetic mutations in Ltv1, and if such resistance can be overcome with drugs that augment destruction of ribosome assembly intermediates via the autophagy pathway.

2. KEYWORDS

Triple negative breast cancer
CK1δ-to-Ltv1 circuit
Ribosome assembly
Breast cancer therapy
Autophagy

3. ACCOMPLISHMENTS

Major Goals and Accomplishments

Given the clear evolutionary selection against CRSIPR/cas9-directed *Ltv1*-deficient TNBC generated by the Karbstein lab (see accompanying report), which are needed to evaluate the effects of phosphorylation defective and phosphor-mimetic Ltv1 mutants on TNBC growth and survival and on the effects of CK1δ inhibitors on these cells, we were not able, at this juncture, to transduce these *Ltv1* knockout cell lines with existing Dox-inducible (pLKO-Tet-on) lentiviruses that will allow for conditional expression of Ltv1, Ltv1-A and Ltv1-D transgenes. Once these hurdles have been overcome, by selection for the slow-growing Ltv1-deficient clones in the presence of the CK1δ inhibitor SR-3029 (see accompanying report) we will transduce these cells with these lentiviruses bearing these Ltv1 transgenes and assess effects of wild type and mutant Ltv-1 on growth, clonogenecity (growth in soft agar), survival, migration and invasion ex vivo, and on tumorigenic potential in vivo using orthotopic (cleared mammary fat pad) xenograft studies. The sensitivity of these 3 Ltv1 cohorts of TNBC to top CK1δ inhibitors will also be assessed.

An important goal of our research team is to test if TNBC tumors can develop resistance to CK1δ inhibitors, and if such resistance involves the acquisition of gain-of-function phosphomimetic mutations in Ltv1 or other compensatory gain-of-function mutations that would rescue ribosome assembly. To this end, by serially transplanting surviving MDA-MB-231 TNBC cells that were first cultured in the presence of EC_{50} doses of the CK1δ inhibitor SR-3029 (in 6-well plates) and then sequentially increasing the doses of the SR-3029, we were able to derive several independent MDA-MB-231-SR-3029-resistant (MDA-MB-231 SRR) cell lines (**Figure 1**). To assess the generality of our findings similar studies generating SR-3029 resistant lines have been performed in other tumor systems (e.g. melanoma and multiple myeloma) that are also highly sensitive to this CK1δ inhibitor.

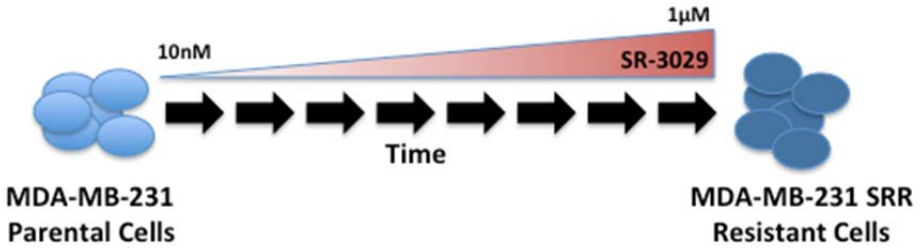

Figure 1. Experimental design for the generation of SR-3029-resistant MDA-MB-231 TNBC cells.

MDA-MB-231 SRR resistant cells were then re-tested for their sensitivity of SR-3029 by performing a standard MTT proliferation assay. As predicted these resistant cells remained refractory to doses of SR-3029 that completely blocked the proliferation of parental MDA-MB-231 cells (**Figure 2**).

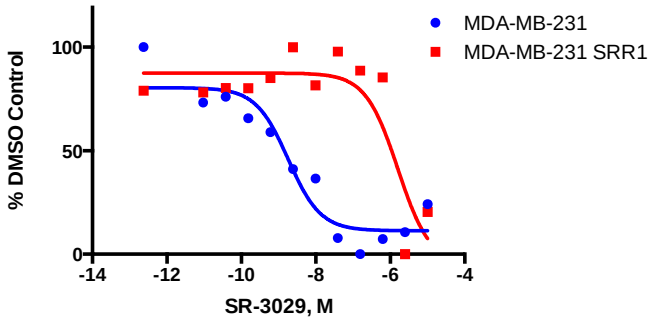

Figure 2. MTT assay of SR-3029-resistant vs. parental MDA-MB-231 cells at the indicated doses of SR-3029

To further chacterize SR-3029 resistant tumor cells we compared their rates of proliferation using standard growth assays. Notably, similar to Ltv1-deficient tumor cells (see accompanying report) SR-3029 resistant tumor cells grew at much slower rates than parental cells (**Figure 3**).

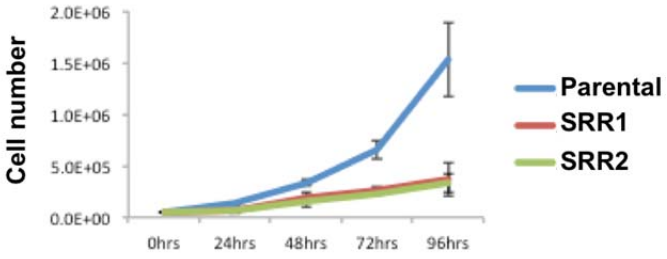

Figure 3. Growth curves were performed with parental vs. SR-3029-resistant tumor cell line and cell counts were performed at the indicated intervals

In TNBC, CK1δ inhibition or silencing disables WNT-β-catenin signaling and the expression of β-catenin/TCF target genes. We therefore isolated RNA from parental and SR-3029 resistant tumor cells and compared their expression of WNT-β-catenin pathway genes using a Nanostring assay of 180 genes in this pathway. Notably, SR-3029 resistant tumor cells displayed markedly different expression of WNT-β-catenin pathway genes than parental tumor cells and the two resistant derivatives also had significant differences in these genes (**Figure 4**); These findings support the notion that there is more than one pathway that can lead to resistance to CK1δ inhibitors and that the selection for resistance does not appear to include events that totally override the inhibitory effects of SR-3029 on the expression WNT-β-catenin pathway genes. We are currently performing RNA-seq analyses of parental versus SR-3029 resistant tumor cells and will assess effects on the expression of Ltv-1 and other ribosome assembly factors.

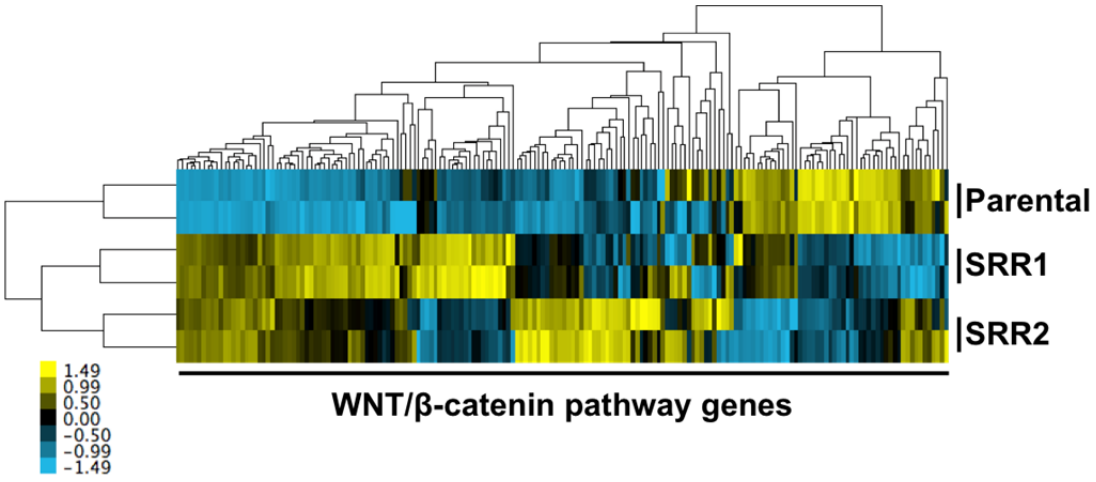

Figure 4. The expression of WNT/β-catenin pathway genes is altered in SR-3029 resistant tumor cells. Nanostring analysis of WNT/β-catenin pathway genes in the indicated tumor cells lines is shown. Genes in the pathway that are up-regulated are in yellow; those that are repressed are in blue.

At this juncture all of our TNBC cell studies, have relied on a single inhbitor (SR-3029) of CK1δ including those showing the gain-of-function Ltv1-D mutant can override the effects of the inhibitor on ribosome assembly. We have therefore screened additional new CK1δ inhibitors obtained from our collaborator Dr. William Roush for their anti-tumor activity in MTT assays. Notably several of these new CK1δ inhibitors (which have been proven as selective CK1δ kinase inhibitors using in vitro kinase assays) have improved potency in MTT assays (**Figure 5**). These

inhibitors will be used to validate the effects of the SR-3029 inhibitor on ribosome assembly and Ltv-1 phosphorylation, and TNBC that are resistant to these inhibitors will also be generated, to determine if resistance is associated with gain-of-function mutations in Ltv1 or in other factors or regulators of ribosome assembly.

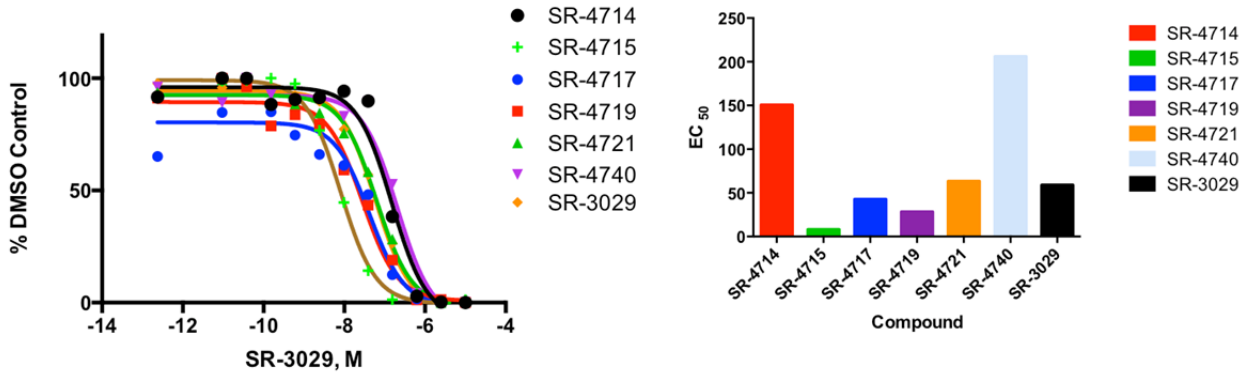

Figure 5. Anti-tumor potency of novel, 3rd generation CK1δ inhibitors. *Left*, EC50 dose response curves with the indicated CK1δ inhibitors. *Right*, EC50 values of the new CK1δ inhibitors *vs*. SR-3029 (black bar).

Training Opportunities and Professional Development

These DOD funded studies allow for training of our new research assistant coordinator (Ms. Dalia Ercan, who replaces Dr Franz Schaub in these studies) in Nanostring technologies and in developing drug resistant tumor cells.

How were the results disseminated?

At this juncture our studies have not been published.

Plans for next reporting period

I. Perform Remaining experiments for Major Task1, and the Studies of Major Task 2 and Major Task 3

(i) CRISPR/cas9-deleted Ltv1-null TNBC clones that have been purified by Dr. Karbstein by growth in the presence of the CK1δ inhibitor SR-3029 (see accompanying report) will be transduced with pLKO-Tet-On-GpNLuc lentiviruses that harbor the reverse tetracycline transactivator ($rtTA^2$) and that are engineered to express wild type Ltv1, Ltv1-A, or Ltv1-D transgenes along with the imaging reporter GpNLuc that was created by the Cleveland lab (an in-frame fusion of eGFP and the small subunit of Nano-luciferase, NLuc). Infected cells will be isolated by FACS for eGFP, and effects of wild type and mutant Ltv-1 on growth, clonogenecity (growth in soft agar), survival (apoptosis assays), migration and invasion, and ribosome assembly (with Dr. Karbstein) ex vivo will be assessed +/- Dox (250 μg/ml, to induce the Ltv1 transgenes). Efficient expression of the Ltv-1 transgenes will be verified by immunoblots and qRT-PCR. The sensitivity of these cells to SR-3029 and other top CK1δ inhibitors will also be assessed in cultures +/- Dox.

(ii) We will assess the tumorigenic potential of Ltv1-deficient vs. replete MDA-MB-231 TNBC, as well as Ltv1-deficient cell engineered to inducibly re-express wild type Ltv1, Ltv1-A, or Ltv1-

D in vivo using orthotopic (cleared mammary fat pad) xenograft studies. Following transplant half of each of these cohorts will be switched to Dox chow to inducibly express the Ltv1 transgenes. Effects on tumor progression will be evaluated by switching to Dox chow 7 days after transplant. Effects on tumor regression will be monitored in cohorts switched to Dox chow when tumor reach 100-mm^3. The sensitivity of these 3 Ltv1 cohorts of TNBC to top CK1δ inhibitors will also be assessed.

II. Fully characterize CK1δ inhibitor-resistant breast cancer cells

(i) Assess resistant TNBC cells for expression/mutation of Ltv1, and for alterations in ribosome assembly factors. Assess ribosome assembly intermediates in these cells with Dr. Karbstein. Repeat these analyses in an additional TNBC cell line, and with a second CK1δ inhibitor. Assess changes in expression via RNA-seq, perform pathway analyses and confirm select differentially expressed genes (e.g., those involved in ribosome assembly) by qRT-PCR, and assess effects on protein levels by immunoblots analyses.

(ii) Assess biological properties (growth, apoptosis, invasion and migration) and tumorigenic potential of CK1δ inhibitor-resistant *vs.* parental TNBC +/- SR-3029 treatment.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project? Our findings support the hypothesis that Ltv1 represents a critical target that when disabled markedly compromises the growth of TNBC cells.

What was the impact on other disciplines? Our analyses of tumor cell lines resistant to CK1δ inhibitors will inform strategies for overcoming resistance, which could be applied to other tumor types treated with such inhibitors.

What was the impact on technology transfer? To date there has been no technology transfer. However, CK1δ inhibitors are being further developed by our collaborator Dr. Roush to produce clinically suitable safety assessment candidates.

What was the impact on society beyond science and technology? Nothing to report at this juncture.

5. CHANGES/PRODUCTS

Changes in approach and reasons for change. Nothing to report at this juncture.

Actual or anticipated problems or delays and actions or plans to resolve them
As noted above, and in the accompanying report of Dr. Karbstein, the generation of the Ltv1-knockout strain of MDA-MB-231 TNBC cells using CRISPR/Cas9 technology proved difficult, as our hypothesis was correct, where Ltv1 is essential for proper growth of TNBC. In particular the very-slow-growth phenotypes of clear Ltv1-null cells is out competed by very minor unedited (i.e., Ltv1 wild type) cells that are still in the culture. However, such Ltv1-null clones are (as would be predicted if Ltv1 were *the* key target) intrinsically resistant to the CK1δ inhibitor SR-3029, allowing us to now select for and grow these cells and kill off wild-type Ltv1 cells. Thus, as soon as these cells are fully purified we are poised to perform the remaining studies of Major Task 1, as well as those of Major Tasks 2 and 3. We will also evaluate the phenotypes of $Ltv1^{+/-}$ TNBC cells, to see if there are effects of *Ltv1* heterozygosity of breast cancer cell growth, survival, invasion and migration, ribosome assembly and tumorigenic potential +/- treatment with SR-3029.

Changes that had a significant impact on expenditures. Nothing to report at this juncture.

Significant changes in use or care of human subjects. Not applicable.

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.

6. PRODUCTS

Website(s) or other Internet site(s): *Nothing to report at this juncture*

Technologies or techniques: *Nothing to report at this juncture*

Inventions, patent applications, and/or licenses: *Nothing to report at this juncture*

Other Products: Nothing to report at this juncture.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	*John L. Cleveland*
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	0.48
Contribution to Project:	*No change*
Funding Support:	*No change*

Name:	Dalia C. Ercan
Project Role:	Research Lab Coordinator
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	6.00
Contribution to Project:	Characterization of CK1-Delta inhibitor resistant breast cancer cells
Funding Support:	*No change*

Name:	Chunying Yang
Project Role:	Research Project Manager
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person month worked:	12.00
Contribution to Project:	Cell Biology and Tumor Xeno graft studies
Funding Support:	*No change*

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

John L. Cleveland, PhD

New Other Support

2R01GM097455-05 (Disney/PI, Cleveland/Co-I) 04/01/16 – 03/31/21 0.6 calendar months
NIH/NIGMS: $35,218 Cleveland/total annual D/C
Title: *Sequence-based Design of Small Molecules Targeting RNA*
Aim 1: Identify drug-like scaffolds that bind RNA
Aim 2: Target disease-associated miRNAs using the lead small molecules identified in Aim 1.
Aim 3a: Develop precision multivalent dimers that allow for selective targeting of miRNA precursors.
Aim 3b: Develop small molecules that target two miRNAs with a single small molecule and study the cellular consequences of dual targeting of disease pathways via designer poly-pharmacy

F32CA203217 (PI:Fernandez/Cleveland-mentor) 04/01/16 – 03/31/18 0.12 calendar months
NIH/NCI $56,042 total annual direct costs (mentor/no salary)
Title: *Role of Branched-Chain Amino Catabolism in Lymphopoiesis and Lymphomagenesis*
Aim 1: What is the role of Bcat1 in B cell lymphopoiesis and homeostasis?
Aim 2: What is the role of Bcat1 in development and maintenance of Myc-induced lymphoma?
Aim 3: What are the metabolic effects of *Bcat1* deficiency in normal and malignant B cells?

6121-1000-00-A (PI:Grass/Cleveland- mentor) 07/01/16 – 06/30/17 0.12 calendar months
RSNA Research & Education Foundation $30,000 total annual direct costs (mentor/no salary)
Title: *Targeting Metabolic Complexes in Small Cell Lung Cancer to Augment Radiosensitivity*
Aim 1: Does SCLC RT resistance require MCT1/CD147-mediated amino acid transport and GSH synthesis?
Aim 2: Can MRSI with hyperpolarized ^{13}C-pyruvate detect *in vivo* efficacy of MCT1 inhibitors?

Completed Research Support

5R01 CA154739 (MPI – Cleveland/Roush) 02/29/12 - 12/31/16
NIH/NCI
Targeting Slc16a/Mct Lactate Transporters in Cancer Therapeutics

What other organizations were involved as partners? *Nothing to report at this juncture*

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:**
  The BCRP Breakthrough DOD_W81XWH-15-BCRP-BREAKTHROUGH-FL12) studies of the laboratories of Drs. Katrin Karbstein (Initiating Principal Investigator [PI], Scripps) and John Cleveland (Collaborating/Partnering PI, Moffitt Cancer Center)

9. APPENDICES: *Nothing to report at this juncture*