AWARD NUMBER:	W81XWH-14-1-0081
TITLE:	CDCA7L and Mechanisms of Increased Male Bias in Glioma
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CONTRACTING ORGANIZATION:	The Geneva Foundation Tacoma WA 98402
REPORT DATE:	May 2017
TYPE OF REPORT:	Annual
PREPARED FOR:	U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012
DISTRIBUTION STATEMENT:	Approved for Public Release; Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	. REPORT TYPE	3. DATES COVERED
May 2017	Annual	15 April 2016 - 14 April 2017

4. TITLE AND SUBTITLE
CDCA7L and Mechanisms of Increased Male Bias in Glioma

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0081

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Geneva Foundation
Tacoma WA 98402

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)
CDMRP

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
We are proposing to study CDCA7L in an NF1 mutant model of astrocytoma and glioblastoma and in neurotransmitter levels in NF1 mutant brains, comparing males and females. The results of his work can be used to develop additional hypotheses on whether a "yin-yang" relationship exists in males and females between risk for brain cancer and risk for depression, or other earning and social dysfunctions. Developing new treatments for gliomas and learning/social dysfunction through a better understanding of the basic biology will benefit both male and female NF1 patients in the long term.

15. SUBJECT TERMS
Nothing listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	8	19b. TELEPHONE NUMBER *(include area code)*

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1. Introduction

CDCA7L has been identified as a candidate modifier of brain tumors in males. Male glioblastoma (GBM) patients with higher expression of *CDCA7L* in their tumor have worse prognosis, and we have shown that overexpression of *Cdca7l* in mouse male and female astrocytes stimulates transformation in male cells, but cell death in female cells. The protein product of *CDCA7L*, R1 represses expression of monoamine oxidase, an important regulator of catecholamines in the brain. This led us to hypothesize that R1 may have a normal function in regulating catecholamine levels, and thus behavior or mood. While males are at an increased risk for GBM, females are at an increased risk of depression and we hypothesize that the role of R1 in behavior and/or mood may also be sex dependent. NF1 patients are at increased risk for both brain cancer and depression, so we are particularly interested in whether mutation in *NF1* and variability in *CDCA7L* at the expression level interact genetically and affect GBM tumorigenesis and/or behavioral changes such as depression. The goal of this project is to better understand the regulation of *CDCA7L* expression and to characterize a *Cdca7l* mutant mouse model, both alone and in combination with NF1 mouse models.

2. Keywords

Neurofibromatosis type 1
CDCA7L
Astrocytoma
Glioblastoma
MAO
Catecholamines
Sex differences
Mouse models

3. Accomplishments

Major Goals and Accomplishments:
In the previous year, we accomplished the following, according to the tasks laid out in the SOW:

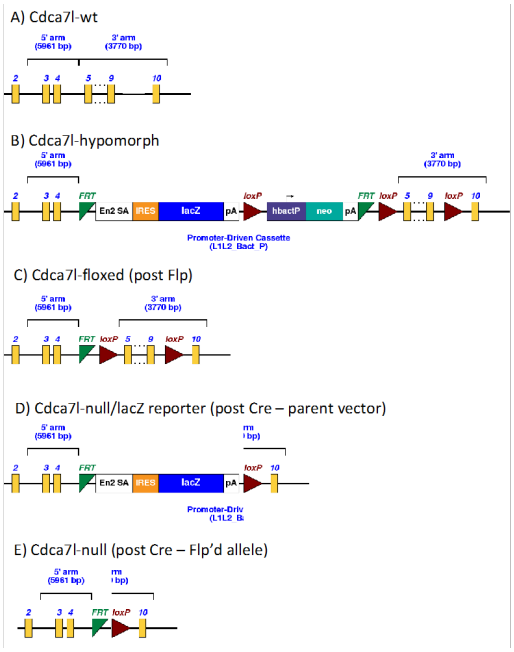

Figure 1: Allele configuration for Cdca7l mutant mice

Task 1: Establish maintenance colonies for C57BL/6N-*Cdca7l-/+*, C57BL/6J-*Nf1-/+;Trp53-/+cis*, C57BL/6J-*Nf1-/+*, and C57BL/6J-*beta-actin-Cre-ERT* for breeders needed in Task 2, 3, 4, and 5.

We continue to maintain colonies of mutant mice as described in the Statement of Work. We have generated the *Cdca7l-null/lacZ* allele (Fig 1D) and have confirmed that it is a null allele using new PCR primers redesigned in the past year (Fig 2). Sequencing the genomic DNA through the allele confirmed the expected configuration.

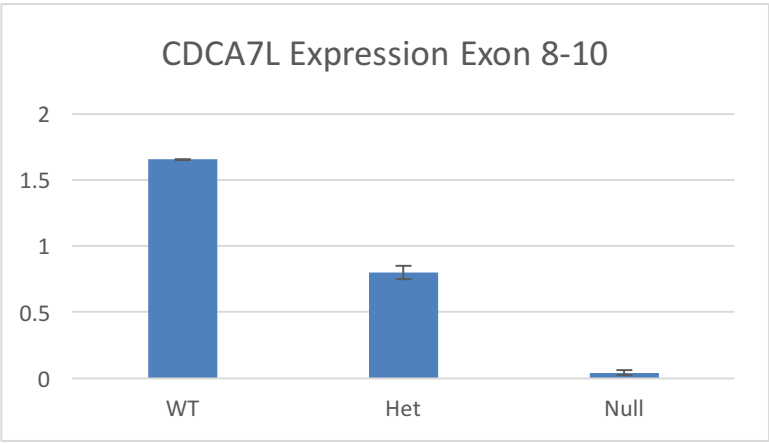

Figure 2: Expression of Cdca7l in mouse brains by RT-qPCR

Task 2: Establish expression pattern of *Cdca7l* in males and females during brain development using a LacZ insertion reporter.

We have been using available *Cdca7l-null* mice to age mice for task 3 and to generate cohorts for task 4 and task 9. Now that these cohorts are near completion, we will begin taking *Cdca7l* mutant mice at different ages to stain histology sections for expression of the LacZ reporter (Fig 1D). Preliminary studies using our core facility for ß-gal staining gave very weak, barely detectable staining, so we expect to need to first optimize the staining protocol.

Task 3: Determine null and heterozygous *Cdca7l* mutant phenotype.

The aging cohorts of *Cdca7l-null/+* and *Cdca7l-null/null* mice we generated in the previous year did not develop any obvious phenotypes in the first 15 months of age. The experiment was ended when the mice were between 15.5 and 15.7 months of age in Feb 2017. We are in the process of analyzing the results, but thus far no anatomical defect has been detected. We have not collected prenatal time points given that the ratio of genotypes in the litters does not suggest any embryonic lethality and no physical defects are present that suggest developmental defects. We have concluded that *Cdca7l* mutants are viable, fertile, and can live to a normal age. We now hypothesize that if these mice have a phenotype, it is likely developmental or acting in cooperation with other mutations. Once the histology slides for this experiment have been reviewed by a qualified pathologist, the characterization of the mutant phenotype will be written up for publication and the mutant mice will be made available to any researchers interested in studying interactions with other genetic mutations.

Task 4: Test the hypothesis that reduced levels of *Cdca7l* inhibit brain tumors in *NPcis* mice.

We have generated cohorts of 10 female and 13 male *NPcis;Cdca7l-null/+* mice that are currently between 7.2 and 10.7 months of age. The median survival of *NPcis* mice in our past datasets was 7.1 months, so we are interested to see if heterozygotic *Cdca7l* mutation inhibits tumorigenesis and whether there is any sex effect. We are continuing to age these cohorts to 12 months and are currently breeding *NPcis;Cdca7l-null/null* cohorts.

Task 5: Identify overlap of SNPs with putative *CDCA7L* regulatory regions.

We recently have taken several complementary approaches to identifying SNPs in regulatory regions using rVISTA and the Genomatix set of tools, now licensed by NCI. We have aligned the human and mouse *CDCA7L-RAPGEF5* intergenic region using both VISTA and DiAlign (Genomatix). mVISTA was used to find aligned regions between mouse and human (Fig 3). rVISTA was used to identify transcription factor binding sites in the human sequence, which we are overlaying on the aligned regions along with SNP data. A total of 913 transcription factor matrices were examined of which ~540 were found

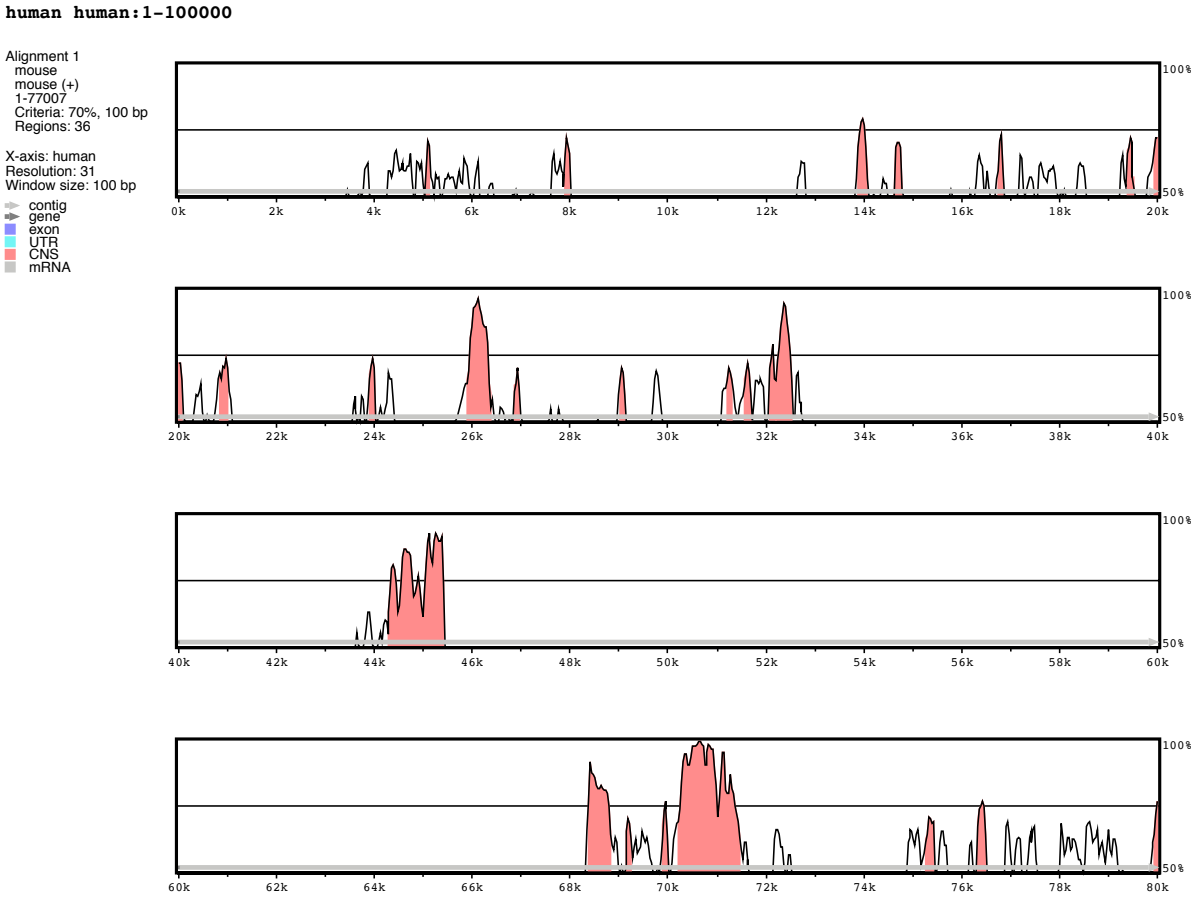

Figure 3: mVISTA alignment of human and mouse intergenic sequence

to have putative recognition sites within the human sequence. In a second method, we isolated the aligned sequences identified in mVISTA and used ModelInspector from Genomatix to identify Modules of Regulatory Elements (MOREs), cassettes of 2 or more transcription factor binding sites with established distance and orientation that are more likely to be biologically significant. In this program, we identified 3 regions where human SNPs and mouse SNPs polymorphic for B6 and 129 were in the same region and potentially disrupted transcription factor binding. These sites bound 1) vitamin D receptor and ELK, 2) STAT5B, IRF4, and E4BP4, and 3) COUP and GATA1. The COUP-GATA1 motif is enriched in genes involved in many biological processes, including neuron dendrite development, negative regulation of apoptosis, and myelination. We are currently analyzing the enrichment of the other identified MOREs. Finally, in a third method, we used the DiAlign program from Genomatix to align the human and mouse sequences *de novo* and identify transcription factor motifs in the aligned regions that are associated with ubiquitous expression and nervous system expression. This analysis identified 63 candidate transcription factors. We are currently aligning SNPs to these results. We plan to compare the alignments and transcription factors identified by these different programs.

Task 6 and 7: As described in the previous annual report, these tasks will be replaced by the direct engineering of cells using CRISPR technology.

Task 8: Test candidate trans-acting regulatory factors in the control of *CDCA7L* expression.

While we are finishing the bioinformatic analysis of the intergenic region, we will investigate the ModelInspector MOREs results directly using siRNA to knockdown expression of VDR, ELK, STAT5B, IRF4, E4BP4, and COUP in GBM cells and astrocytes and look at changes in *CDCA7L* expression. These transcription factors are predicted to have human SNPs within their binding sites that interrupt the binding site consensus sequence. We are planning these experiments now and expect to complete them in the next 6 months.

Task 9: Examine the interaction of changing *Cdca7l* levels with *Nf1* mutation on brain phenotypes, catecholamine levels, and response to dopamine pathway therapeutics

We have established a subcontract with Michigan State University to analyze catecholamine levels in different brain regions and are perfecting the brain dissection technique required for the analysis. We will analyze adult mouse brains first, then neonatal brains. The adult crosses between *Nf1* mutants and *Cdca7l* mutants have been completed, and we expect to have samples ready to send to MSU in the next month or two.

Opportunities for Training and Professional Development:

Mr. Mackenzie Silverman has been supported by this award during this reporting period. Mr. Silverman has learned many new techniques during his post-baccalaureate fellowship this year and has generated data that will be used in a publication on *CDCA7L*. He participates in seminar series and group meetings within the Pediatric Oncology Branch and is schedule to give a talk on his work on June 26, 2017. Last fall he attended the Society for Neuroscience annual meeting in San Diego, exposing him to a wide variety of research in neuroscience, which he is planning to pursue in medical school. He was accepted into the medical school program at Thomas Jefferson University and will matriculate in the fall.

Dissemination of Results:

We are still revising our manuscript on the sex-specific function of *CDCA7L* in tumorigenesis and Mr. Silverman has made progress on the mechanism of *CDCA7L* action. As mentioned above, Mr. Silverman will present his data at a department seminar in June for broader feedback. Our major goals have not yet reached the maturity for publication, although we plan to submit the characterization of the *Cdca7l* mutant mouse phenotype as soon as the pathology analysis is completed.

Plans for Next Reporting Period:

In the next reporting period, I will hire another post-baccalaureate fellow to replace Mr. Silverman when he goes to medical school. This new post-baccalaureate fellow will complete the project. Due to the academic calendar, I anticipate requesting a final extension of approximately 4 months to allow the fellow to complete the work and have a full year of fellowship training.

We plan to test regions and transcription factors for their role in *CDCA7L* regulation in the next year, using siRNA knockdown of transcription factors and CRISPR modification of SNPs in transcription factor binding sites. This will help us understand whether genetic variation in *CDCA7L* regulation may contribute to the incidence of GBM and/or depression.

Data from our *NPcis;Cdca7l* cross will be analyzed to determine whether reduction in *Cdca7l* expression levels can affect brain tumorigenesis. Catecholamine levels from *Cdca7l* mutant brains and *Nf1;Cdca7l* mutant brains will be analyzed to determine whether reduction in *Cdca7l* has a potential role in catecholamine-related diseases such as depression, and whether there is any interaction with *Nf1* mutation that might suggest an intensified effect in NF1 patients.

We plan to prepare manuscripts of this data in the next reporting period to disseminate our findings publically and make our *Cdca7l* mutant mouse available to other researchers.

We expect that we will need to request a no cost extension in the next reporting period.

4. Impact:

We have nothing new to report this period.

5. Changes/Problems:

Because I am only able to hire short term fellows in the remainder of the grant funding period, I will have another staff turnover this summer that will slow the project down somewhat, depending of the skills of the incoming fellow.

We discovered in the past reporting period that the RT-PCR primers we had been using to examine *Cdca7l* expression in the mouse were in a region of the genome that overlapped another gene *Dnah11* that is in a tail-to-tail orientation with *Cdca7l* such that the 3' ends overlap. To ensure we were not detecting any *Dnah11* transcript, we redesigned the *Cdca7l* expression primers to overlap exon-intron boundaries. The redesigned primers are cleaner and have helped us verify the lack of expression of *Cdca7l* in the null mice, which we have tested using multiple primer sets. Because of the sequence in the region, this primer redesign was unfortunately not straightforward and delayed our characterization of the mouse model.

In addition to primer issues, we had been optimizing protein techniques using a polyclonal goat antibody from Santa Cruz and recently learned that Santa Cruz has shut down all its polyclonal antibody production, and has not yet developed a comparable monoclonal for *CDCA7L*. Mr. Silverman has done extensive work to characterize all the commercially available *CDCA7L* antibodies and has found one that works fairly well in our assays. This has also cost us unanticipated time and money.

6. Products:

Research Materials: *Cdca7l* null mice

7. Participants and Other Collaborating Organizations:

Name:	Mackenzie Silverman
Project Role:	Post-baccalaureate Fellow
Researcher Identifier:	
Nearest person months worked:	11
Contribution to the project:	Mr. Silverman has been working on the mechanism of how CDCA7L functions at the transcriptional level. He has developed the approach to testing catecholamine levels and has been examining the phenotype of *Cdca7l* mutant mice. He helped with the alignment of transcription factor binding sites to aligned regions between mouse and human upstream of *CDCA7L*. Mr. Silverman's salary is provided by this award. Mr. Silverman will leave the project on June 26, 2017 prior to beginning medical school.

Name:	Karlyne Reilly
Project Role:	Principal Investigator
Researcher Identifier:	0000-0001-9109-4409
Nearest person months worked:	3

Contribution to the project:	Dr. Reilly has monitored the mouse colony, determining which breeders to set-up and which mice to euthanize for analysis. She has also managed the budget, written the animal study protocols and modifications, and supervised Mr. Silverman and Mr. Tuskan. She has reviewed slides from *Cdca7l* mutant mice. She has performed the bioinformatics analysis of the regulatory region upstream of *CDCA7L*. Dr. Reilly's salary is provided by the National Cancer Institute.
Name:	Robert Tuskan
Project Role:	Technician
Researcher Identifier:	
Nearest person months worked:	2
Contribution to the project:	Mr. Tuskan has prepared tail DNA from the *Cdca7l* mouse colony and genotyped them for whether they carry the *Cdca7l* mutation. Mr. Tuskan has designed primers to sequence the *Cdca7l* allele construct. Mr. Tuskan also helps to monitor the budget and places all orders for the grant. Mr. Tuskan's salary is provided by the National Cancer Institute.
Organization Name:	Leidos
Location:	Frederick, MD
Project Role:	Animal Technical Support and Histology Technical Support
Nearest person months worked:	2
Contribution to the project:	The animal technical support staff at NCI, Frederick monitor the health of the mouse colony, set up breeders, tail clip mice, and euthanize mice under Dr. Reilly's instruction. The histology technical support at NCI, Frederick, euthanize and dissect mice as needed for the project and process tissues for histology. Slides are sent to Dr. Reilly for review.

A subcontract agreement has been established with Michigan State University to perform catecholamine analysis, but no work has begun on this contract.

Changes in active support: Nothing to Report.