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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.

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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 revised SOW dated 27-Jan 2017:

Task 1: Establish expression pattern of *Cdca7l* in males and females during brain development using a LacZ insertion reporter.

The mouse cohorts to examine LacZ expression have been collected and we are working to develop immunohistochemistry for ß-galactosidase on formalin-fixed sections, due to technical issues with staining for the ß-galactosidase enzymatic activity.

Task 2: Determine null and heterozygous *Cdca7l* mutant phenotype.

We have not yet found any anatomical defects in *Cdca7l-/+* or *Cdca7l-/-* adult mice, but are still in the process of having a qualified pathologist review the slides. Once the slides have been read, the phenotype will be written up as a publication and the mutant mice will be made available to any researchers interested in studying interactions with other genetic mutations. Recent data on catecholamines (see Task 7) suggests an interaction with *Nf1* mutation in females and we are following up this observation with additional experiments.

Task 3: Test the hypothesis that reduced levels of *Cdca7l* inhibit brain tumors in *NPcis* mice.

As described in our recent request for a NCE, we are encountering unexpected results for this task in that our *NPcis* mice in the absences of any *Cdca7l* mutation are not developing tumors. New breeding pairs from our Bethesda colony are being imported to the Frederick colony and are expected to be released from quarantine by the end of this week. We will set up new breeders to verify the results.

Task 4: Identify overlap of SNPs with putative *CDCA7L* regulatory regions.

We have prioritized 8 regulatory sites upstream of *CDCA7L* (Fig 1; Table 1 and 2), with the highest priority being a TCF4/TAL1B binding site. TCF4 is downstream of Wnt/ß-catenin signaling and is involved in initiation of neuronal differentiation. In addition, in a gene expression array of mouse tumors TCF4 correlated negatively to *Cdca7l* levels in males (r^2=-0.63; P=0.039) and positively to *Cdca7l* levels in

females (r^2=0.83; P=0.0053). In a Cox model analysis of RNAseq data from GBM in TCGA, TCF4 showed a significant interaction of expression level with the sex of the patient on the survival time. Taken together, these sources of data point to TCF4 as a potential candidate for playing a role in sex differences in brain cancer, thus we are looking at TCF4 first as a potential regulator of CDCA7L and its background-specific, differential effects on males and females.

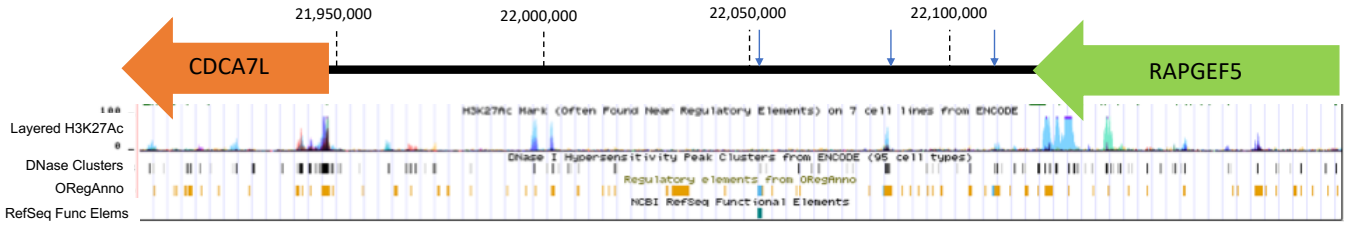

Figure 1: CDCA7L-RASGEF5 intergenic region. Blue arrows indicate the locations of prioritized regulatory sites. UCSC tracks below the diagram show H3K27Ac data from ENCODE, indicative of regulatory regions; DNase I hypersensitivity peaks from ENCODE; regulatory elements from ORegAnno; and functional elements from NCBI RefSeq.

Table 1: Select Human SNPs on Conserved TF binding sites between CDCA7L and RAPGEF5

Chr	TFBS.start	TFBS.end	TF	orient	Position.SNP	SNP.ID	
chr7	22091479	22091495	S8	+	22051867	rs73067060	PRRX2
chr7	22091483	22091494	PAX4	-	22051876	rs73067060	PAX4
chr7	22115078	22115094	MEF2	+	22075475	rs115069027	MEF2A/B/C/D
chr7	22115284	22115312	PAX5	+	22075667	rs118000245	PAX5
chr7	22127590	22127620	HOX13	-	22087980	rs12534112	HOXA13 and HOXD13
chr7	22127714	22127729	CDPCR3	-	22088110	rs2158878	CUX1
chr7	22150075	22150091	TAL1ALPHAE47	+	22110473	rs17146221	TAL1A and TCF3
chr7	22150075	22150091	TAL1BETAITF2	+	22110473	rs17146221	TAL1B and TCF4

Table 2: Select Mouse 129S1/B6 SNPs on Conserved TF binding sites between Cdca7l & Rapgef5

Chr	TFBS.start	TFBS.end	TF	orient	Position.SNP	SNP.ID (internal)
chr12	117805926	117805942	S8	+	117805936	A/T_129S1_SvImJ
chr12	117805926	117805942	S8	+	117805929	A/G_129S1_SvImJ
chr12	117805926	117805942	S8	+	117805927	G/T_129S1_SvImJ
chr12	117805926	117805942	S8	+	117805931	C/T_129S1_SvImJ
chr12	117805930	117805941	PAX4	-	117805936	A/T_129S1_SvImJ
chr12	117805930	117805941	PAX4	-	117805931	C/T_129S1_SvImJ
chr12	117805930	117795270	(42 not seen in human data)			
chr12	117793464	117793486	MEF2	-	117793479	A/G_129S1_SvImJ
chr12	117793464	117793486	MEF2	-	117793485	A/G_129S1_SvImJ
chr12	117786847	117786877	HOX13	-	117786877	T/G_129S1_SvImJ
chr12	117786800	117786820	(3 not seen in human data)			
chr12	117786787	117786815	PAX5	-	117786807	T/C_129S1_SvImJ
chr12	117786787	117786815	PAX5	-	117786794	A/T_129S1_SvImJ
chr12	117786235	117775884	(3 not seen in human data)			
chr12	117774847	117774863	MEF2	-	117774857	G/T_129S1_SvImJ
chr12	117774622	117772864	(8 not seen in human data)			
chr12	117771254	117771269	CDPCR3	+	117771262	G/T_129S1_SvImJ
chr12	117771188	117770415	(6 not seen in human data)			
chr12	117764987	117765003	TAL1ALPHAE47	-	117764991	C/T_129S1_SvImJ
chr12	117764987	117765003	TAL1BETAITF2	-	117764991	C/T_129S1_SvImJ

Task 5: Use CRISPR technology to mutate putative regulatory SNPs in 2 male and 2 female GBM lines to look at effect on *CDCA7L* expression.

We have researched CRISPR technology and are expanding the 4 GBM lines to be used in this task. CRISPR experiments will be conducted in the next year. We will start with the alteration of SNP

rs17146221 at chr 7:22110473 in the human genome.

Task 6: Test candidate trans-acting regulatory factors in the control of *CDCA7L* expression.

We have been developing the reagents needed to test the role of TCF4 in *CDCA7L* expression. PCR primers to detect the expression level of TCF4 by RT-qPCR have been developed (Fig 2) and the transfection conditions for siRNA in GBM cells has been optimized using the siKiller system. We are currently optimizing TCF4-specific siRNAs. Currently, we have achieved 60% knockdown of TCF4 and are trying to improve the knockdown level with different siRNA constructs. Once the knockdown has been optimized, we will examine the effect on *CDCA7L* expression levels and cell growth rate in male and female cells.

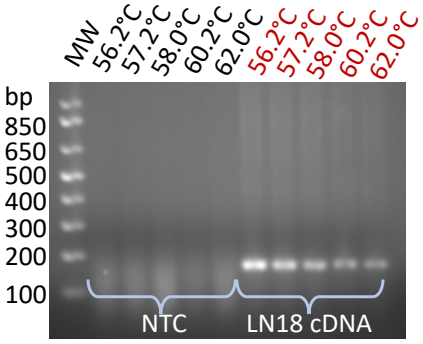

Figure 2: Temperature gradient to test qPCR primers for TCF4 expression.
NTC = no template control

Task 7: Examine the interaction of changing *Cdca7l* levels with *Nf1* mutation on brain phenotypes, catecholamine levels, and response to dopamine pathway therapeutics

We have dissected striatum, amygdala, hippocampus, and prefrontal cortex from 16 female and 14 male mice of different genotypes, and analyzed catecholamine levels through a subcontract with Michigan State University. Unfortunately, the data could not be combined with our previous catecholamine data from last year (we are currently looking into why that is), but there seemed to be less variability in the second experiment (although variability was still high in some brain regions and some catecholamines). We saw significant interaction of *Nf1* and *Cdca7l* mutations on the levels of norepinephrine and 5-HT in the amygdala in females, but not males (Fig 3). Because of the variability, likely due to the dissection technique or individual mouse variation, we are looking into additional ways of quantifying catecholamines in different brain regions to verify male-female differences.

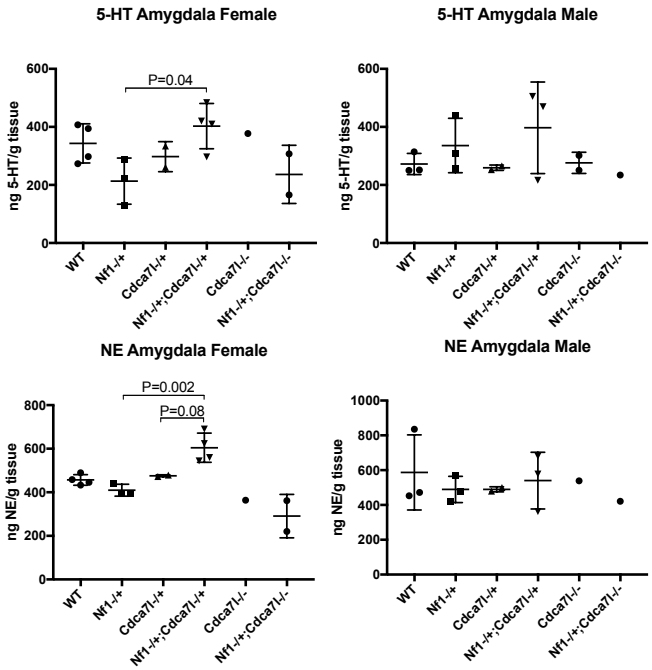

Figure 3: Levels of 5-HT (top) and norepinephrine (NE) (bottom) in dissected amygdalas from female (left) and male (right) mouse brains of different genotypes.

Opportunities for Training and Professional Development:

Mr. Mackenzie Silverman and Ms. Impana Shetty were both post-baccalaureate trainees supported by this award. Mr. Silverman left the lab shortly into this reporting period and has started his medical training at Thomas Jefferson University. Ms. Shetty has presented her work as a poster at a departmental retreat and at the NIH Post-Bac day. Ms. Katie Pendo is a technician supported by this award and has been very proactive about taking advantage of training opportunities at NIH, most recently training in methods of mouse genetics and mouse handling that will allow her to take a greater role in the phenotyping of the *Cdca7l* mutant mouse model in the upcoming year. All of the members of the lab supported by this award are actively involved in the departmental seminar series and group meetings within the Pediatric Oncology Branch. Mr. Silverman presented his work in an oral presentation to the Pediatric Oncology Branch in June 2017, and it is planned that both Ms. Shetty and Ms. Pendo will give talk in late 2018.

Dissemination of Results:

We are still revising our manuscript on the sex-specific function of *CDCA7L* in tumorigenesis and

Ms. Pendo is continuing experiments from Mr. Silverman. As mentioned above, Ms. Shetty presented a poster of her work at a departmental retreat. 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, we will complete the experiments described in the revised SOW, and expect this will be the last year of the award. Specifically, we will finish characterizing the *Cdca7l* mutant mouse phenotype and the interaction of *Cdca7l* mutation with *Nf1* mutation and *Nf1;Trp53* combined mutation. We will complete testing the role of TCF4 and TCF4 binding sites in the regulation of *CDCA7L* expression and will work through as many of the other sites/factors listed in Table 1 as time permits. We will examine more closely the structure of the amygdala in *Cdca7l* mutant brains, and develop *in situ* techniques to look at catecholamine and/or *Mao* levels in this brain region. At the end of the next reporting period we plan to write up our results for publication

4. Impact:

We have nothing new to report this period.

5. Changes/Problems:

We have had a lot of construction in our mouse facility that may affect the breeding and phenotypes of the mice. The moves of mouse cages should be finished for the rest of the award period, so we are hopeful that the colony will be more stable going forward. The analysis of the catecholamine levels has been challenging because of variability in the dissection technique and the number of different genotypes to analyze. I had expected to be able to combine the ng catecholamine/g tissue values to increase the sample size between the two runs, but in most of the brain regions the values were 10-fold different in the 2 runs. I am looking into why this was the case. Using a complementary technique to quantify catecholamines would increase confidence in the differences we see. We are looking into several recently developed technologies available in the Center for Cancer Research to resolve this issue.

6. Products:

Research Materials: *Cdca7l* null mice

7. Participants and Other Collaborating Organizations:

Name:	Katie Pendo
Project Role:	Technician
Researcher Identifier:	
Nearest person months worked:	8
Contribution to the project:	Ms. Pendo has worked on the mechanism of *CDCA7L* regulation of gene expression and on the effects of *CDCA7L* downregulation. She is developing CRISPR protocols for our group to modify regulatory regions upstream of *CDCA7L.* She is responsible for maintaining cell lines for the project. She also helps to monitor the budget and places all orders for the grant. Ms. Pendo's salary is provided by this award.
Name:	Impana Shetty
Project Role:	Post-baccalaureate Fellow
Researcher Identifier:	
Nearest person months worked:	8
Contribution to the project:	Ms. Shetty has worked on identifying regulatory regions upstream of *CDCA7L*. She is responsible for using siRNA techniques to knockdown TCF4, a top candidate for genetic background-specific regulation of *CDCA7L*, and look at the effect on *CDCA7L* expression. Ms. Shetty's salary is provided by this award.

Name: Mackenzie Silverman
Project Role: Post-baccalaureate Fellow
Researcher Identifier:
Nearest person months worked: 2
Contribution to the project: Mr. Silverman developed the approach to testing catecholamine levels and has been examining the phenotype of *Cdca7l* mutant mice. Mr. Silverman's salary is provided by this award. Mr. Silverman left 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, Ms. Pendo, and Mr. Tuskan. She has reviewed slides from *Cdca7l* mutant mice. She has performed the bioinformatics analysis of the regulatory region upstream of *CDCA7L*. She performed dissections to examine the levels of catecholamines in different brain regions in wild-type and *Cdca7l* mutant mice. 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.

Changes in active support: Nothing to Report.