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TITLE: Interrogation of the KDM1A-IL18 Axis in Pediatric High-Grade Glioma

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14. ABSTRACT

Diffuse Intrinsic Pontine Glioma (DIPG) is a type of high-grade glioma that remains uniformly fatal in 2022, represents the leading cause of death for pediatric brain tumor patients, and for which the standard of care has not improved in over 50 years. In addition, despite at least 100 clinical trials, there is not a single drug that has been shown to significantly prolong the survival of children with DIPG beyond radiation alone.The objective of our study is to unravel the role of LSD1 or KDM1A in DIPG pathogenesis focusing on its effect on immune related genes. We hypothesize that LSD1 promotes gliomagenesis in part by repressing immune-related genes such as IL18, and that inhibition of KDM1A will be efficacious in prolonging the survival of DIPG-bearing mice, in part through upregulation of IL18, thereby altering the tumor microenvironment. At the end of year 1, we have made progress toward accomplishing the Statement of Work and our data suggests that LSD1 is a bone-fide therapeutic target in DIPG. In the upcoming two years we will clarify the mechanism and prioritize a drug and potentially a combinatio for clinical translation.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

   Diffuse Intrinsic Pontine Glioma (DIPG) is a type of high-grade glioma that remains uniformly fatal in 2020, represents the leading cause of death for pediatric brain tumor patients, and for which the standard of care has not improved in over 50 years. In addition, despite at least 100 clinical trials, there is not a single drug that has been shown to significantly prolong the survival of children with DIPG beyond radiation alone.The objective of our study is to unravel the role of LSD1 or KDM1A in DIPG pathogenesis focusing on its effect on immune related genes. We hypothesize that LSD1 promotes gliomagenesis in part by repressing immune-related genes such as IL18, and that inhibition of KDM1A will be efficacious in prolonging the survival of DIPG-bearing mice, in part through upregulation of IL18, thereby altering the tumor microenvironment.

2. **KEYWORDS:** high grade glioma, LSD1, KDM1A, histone demethylase, DIPG

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.*

   - **What were the major goals of the project?** *List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project identify these dates and show actual completion dates or the percentage of completion.*
   - **Specific Aim 1**: Determine the effects of LSD1 deletion and overexpression in DIPG pathogenesis in vivo in genetic and xenograft models
   - **Specific Aim 2 -**Determine whether IL18 is a direct target gene of LSD1 and elucidate the function of IL18 in DIPG pathogenesis using both genetic and xenograft models of DIPG
   - **Specific Aim 3** Determine the efficacy of LSD1 inhibitors in genetic and xenograft models of DIPG.

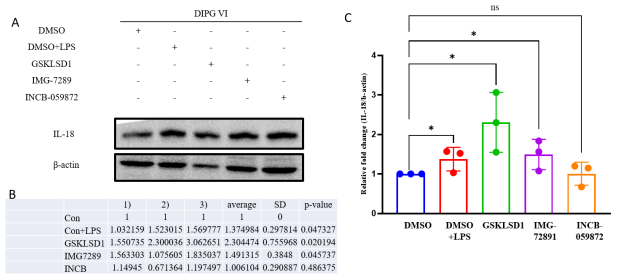
   - **What was accomplished under these goals?**

     - *For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.*

- We have completed the in vitro assessment of three LSD1 inhibitors in 4 DIPG cell lines: DIPG IV, DIPG VI, DIPG 10 and DIPG XIII. Three LSD1 inhibitors were evaluated: GSK LSD1, IMG-7289 (bomedemstat), and INCB-059872. Dose response data shown in Table 1 indicates that made the DIPG lines were relatively insensitive to INCB-059872, with an IC50 not reached in three of the four lines tested. The greatest potency was seen with IMG-7289 which had the lowest IC50 values across all four lines compared to the other two inhibitors. In Figure 1 we examined if IL-18 protein expression was changed by LSD1 inhibition, consistent with experiments proposed in Aim 2. We found that GSK-LSD1 and IMG-7289

IC50	GSKLSD1	IMG-7289	INCB-059872
DIPG IV	134.0 (R2=0.9574)	72.55 (R2=0.7131)	Not to reach
DIPG VI	232.1 (R2=0.6341)	99.25 (R2=0.7765)	194.4 (R2=0.5558)
VUMC-DIPG10	71.88 (R2=0.5987)	40.41 (R2=7240)	Not to reach
DIPG XIII	1270 (R2=0.2359)	173.5 (R2=0.5939)	Not to reach

Table 1. IC50 data for three LSD1 inhibitors in four patient derived DIPG cell lines.



B

	1)	2)	3)	average	SD	p-value
Con	1	1	1	1	0	
Con+LPS	1.032159	1.523015	1.569777	1.374984	0.297814	0.047327
GSKLSD1	1.550735	2.300036	3.062651	2.304474	0.755968	0.020194
IMG7289	1.563303	1.075605	1.835037	1.491315	0.3848	0.045737
INCB	1.14945	0.671364	1.197497	1.006104	0.290887	0.486375

Figure 1. IL-18 protein expression is increased in DIPG cells treated with GSK-LSD1 and IMG-7289. (A) Representative western blot of IL-18 and b-actin in DIPG VI cells treated with LSD1 inhibitors (500 uM, 50 uM and 250 uM for 24 h) or 5 ug/ml LPS for 24h. (B) Densitometric and (C) Graphical representation of three independent experiments.

caused increased protein expression of IL-18 whereas INCB-059872 did not (Fig.1A-C). This was carried out by Western blotting on the DIPG VI cell line (Fig. 1A) and repeated in triplicate as quantified by densitometry and shown graphically (Fig. 1B&C). As a positive control for IL-18 induction, we treated cells with LPS (lipopolysaccharide) and saw a statistically significant increase in IL-18 expression comparable to that seen with a 24 h exposure to IMG-7289 but not as robust as GSK-LSD1 exposure. To determine whether LSD1 inhibition raised transcript levels of IL-18, we treated two DIPG cell lines (DIPG IV and VI) with IMG-7289 and conducted qPCR experiments using IL-18 specific primers (Fig. 2A). A two fold change in IL-18 transcript was seen in both cell lines with a 24 h exposure to IMG-7289 (Fig. 2A). Primers used for IL18 were: AAGATGGCTGCTGAACCAGT (forward) and GAGGCCGATTTCCTTGGTCA (reverse). For PPIA, primers were CCCACCGTGTTCTTCGACATT (forward) and GGACCCGTATGCTTTAGGATGA (reverse).

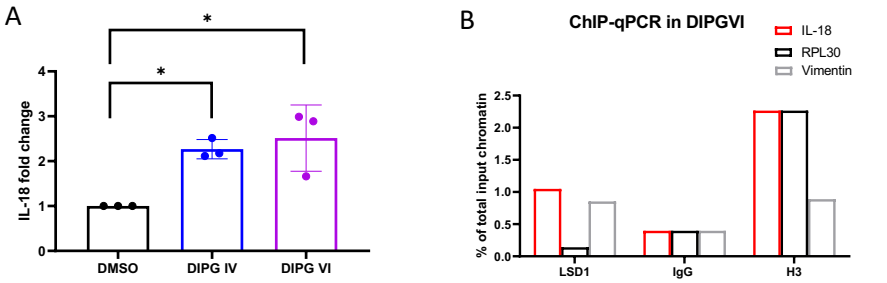


Figure 2. IL-18 transcript and promoter binding. (A) Quantitative real-time PCR (qRT-PCR) of IL-18 mRNA was carried out in DIPG IV and VI cells treated with 45 uM IMG-7289 for 24 hr. Control primer was the housekeeping gene PPIA and served as the loading control for fold change calculations. (B) Chromatin immunoprecipitations were performed with cross-linked chromatin from DIPG-VI cells and either LSD1 Antibody (ab129195) or Normal Rabbit IgG #2729 or Histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated) #4620. The enriched DNA was quantified by real-time PCR Human IL-18 gene promoter regions, and human PRL-30 or Vimentin promoter primers as positive control genes. The amount of immunoprecipitated DNA in each sample is represented as signal relative to the total amount of input chromatin, which is equivalent to one.

These data support aim 2 of the proposal in suggesting that IL-18 protein upregulation is correlated with cytotoxicity of LSD1 inhibition. Also, relevant to aim 2, we have conducted chromatin immunoprecipitation assays showing in the DIPG VI cell line that LSD1 is bound to the IL-18 promoter (Fig. 2B). Collectively these data are supportive of our hypothesis, that LSD1/IL-18 interactions are regulating DIPG growth and proliferation and later this month we will commence an in vivo experiment testing efficacy of GSK-LSD1 and IMG-7289 in an orthotopic xenograft DIPG model, as described in Aim 3 of the proposal. We are also evaluating therapies to enhance efficacy of LSD1 inhibition through the use of small molecules and radiotherapy.

In addition, we completed analysis of the in vivo overexpression experiment we reported in last year's annual report. Below are the final data for this experiment. To summarize, we over-expressing WT LSD1, a truncated LSD1 mutant lacking the C-terminus domain with the demethylase domain, and empty vector in our genetic mouse model of DIPG driven by PDGFB; H3.3K27M and p53 loss. Expression of a truncated LSD1 mutant lacking the C-terminus domain with the demethylase domain resulted in a significantly reduced tumor incidence (**Figure 3**). Immunostaining the cohorts for Ki-67, Sox2, CD44, Nestin, GFAP, Tuj1, MBP, Cleaved Caspase 3, TUNEL, did not show significant differences across the cohort (**Figure 5** shows the quantification of Ki-67 across the cohort). There was a significantly reduced tumor incidence of the LSD1 mutant relative to both the LSD1

WT and empty vector suggesting that the LSD1 mutant may interfere with tumor initiation (**Figure 3, 4, and 5**). We conclude that overexpression of a truncation mutant of LSD1 lacking the demethylase domain significantly reduces tumor initiation. Once tumors form, they are indistinguishable between cohorts.

An important experiment that is still ongoing is the LSD1 deletion experiments using conditional LSD1 mice provided by Michael Rosenfeld. We have induced murine DIPGs with and without LSD1 deletion and those experiments are ongoing. We expect to complete this key experiment including subsequent analysis in year 3.

As part of our investigations of the relationship between LSD1 and IL18, we also studied the effects of IL18 overexpression in vivo. Interestingly overexpression of IL18 accelerates gliomagenesis (**Figure 6**). Lastly, in the past year we also performed a CUT&RUN experiment and noted that there is an LSD1 peak in the IL18 regulatory region. This strengthens our data that LSD1 regulates IL18 levels directly but requires further validation (**Figure 7**).

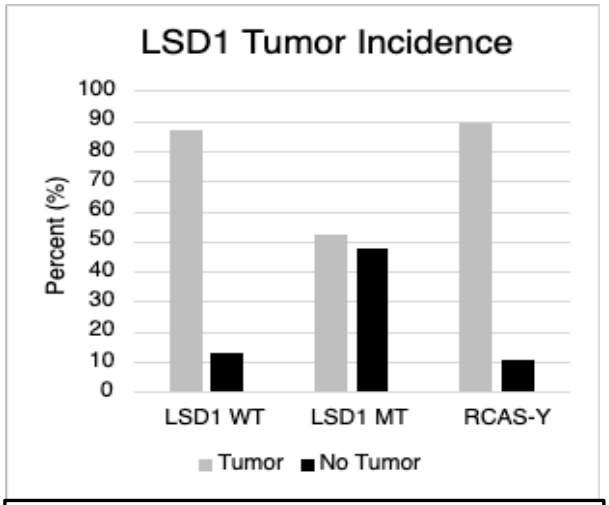


Figure 3. Bar graph of tumor incidence for mice injected with the LSD1 WT, LSD1 MT, and RCAS-Y constructs. There was a significant difference in tumor incidence between LSD1 WT and LSD1 MT cohort:. The two-tailed P value equals 0.0230. There was a significant difference in tumor incidence between LSD1 MT and empty vector. The two-tailed P value equals 0.0045. We conclude that LSD1 MT overexpression interferes with tumor initiation.

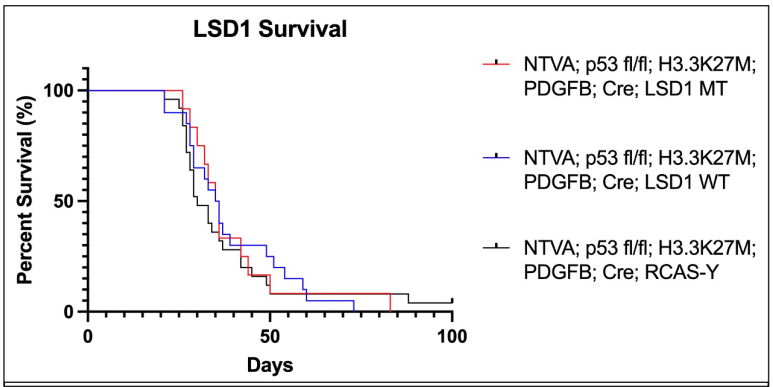


Figure 4. Kaplan-Meier survival curve of tumor-bearing mice injected with LSD1 WT, LSD1 MT, and RCAS-Y constructs.

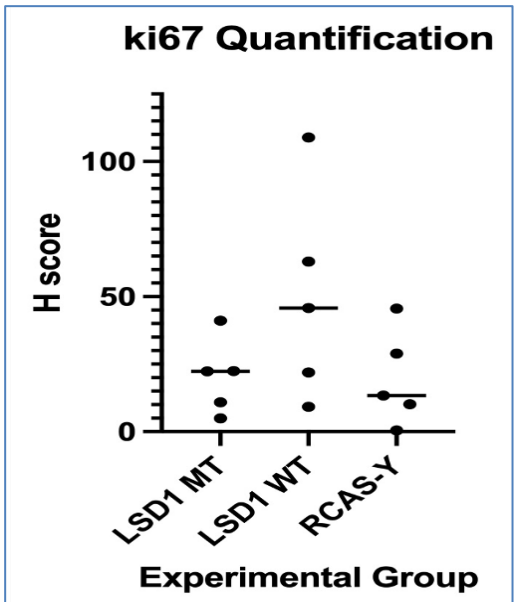


Figure 5. Column scatter graph displaying the H-score comparison of ki67 expression (proliferation) across representative tumors from the three cohorts.

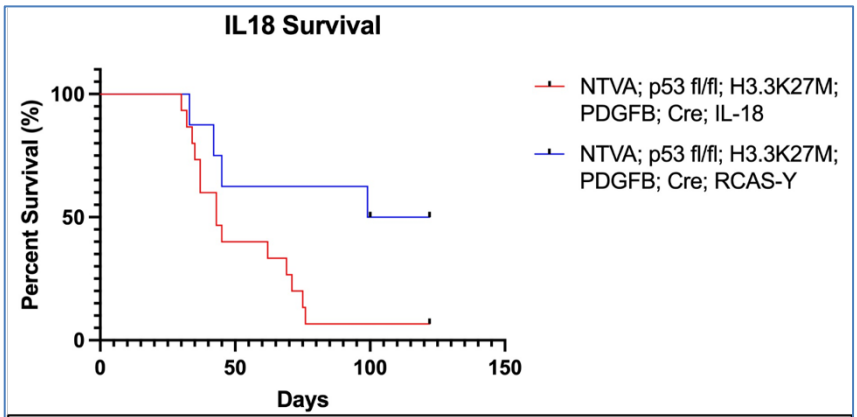


Figure 6. Kaplan-Meier survival curve of tumor-bearing mice injected with IL-18 and RCAS-Y constructs. IL-18 (n=15) significantly accelerates tumorigenesis relative to empty vector (n=8). Log Rank test p<0.0281*.

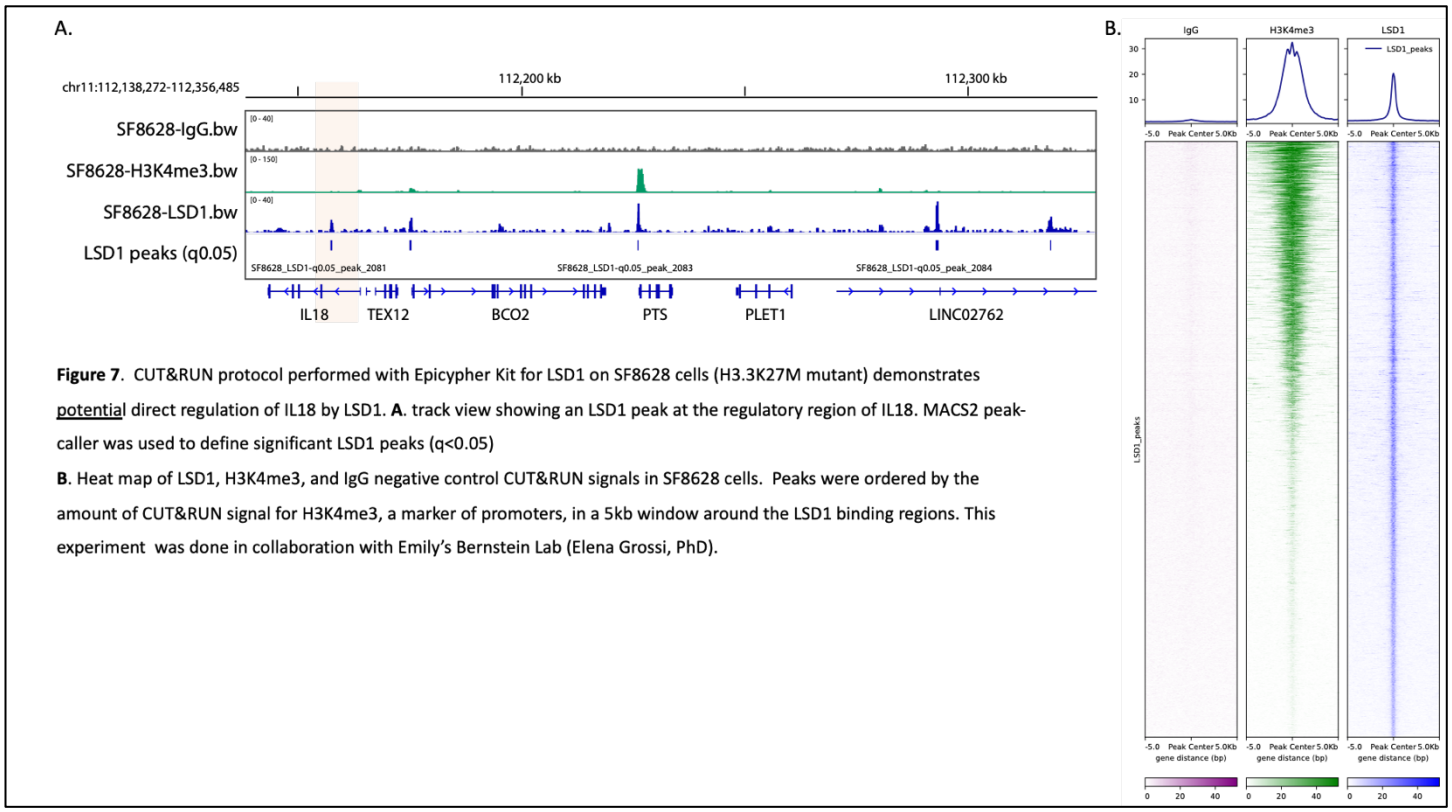


Figure 7. CUT&RUN protocol performed with Epicypher Kit for LSD1 on SF8628 cells (H3.3K27M mutant) demonstrates potential direct regulation of IL18 by LSD1. **A**. track view showing an LSD1 peak at the regulatory region of IL18. MACS2 peak-caller was used to define significant LSD1 peaks (q<0.05)

B. Heat map of LSD1, H3K4me3, and IgG negative control CUT&RUN signals in SF8628 cells. Peaks were ordered by the amount of CUT&RUN signal for H3K4me3, a marker of promoters, in a 5kb window around the LSD1 binding regions. This experiment was done in collaboration with Emily's Bernstein Lab (Elena Grossi, PhD).

Thus, we conclude that the relationship between LSD1 and IL18 is important to better understand as in vivo IL18 promotes tumorigenesis and LSD1 inhibition in vitro also increases IL18. We excited to determine if LSD1 deletion in vivo will impact IL18 levels and we will pursue CUT&RUN in additional DIPG cell-lines in year 3. This highlights the complexity of the role of LSD1 in DIPG, and perhaps it is not surprising as LSD1 is known to be part of complexes that both activate and inhibit transcription.

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

- Funds were utilized to support laboratory personnel carrying out the experiments shown above, who had no prior experience with primary patient derived brain tumor cell lines. This enhanced their skill and prompted them to attend seminars relevant to brain tumor therapeutics. This funding supported reagent costs and supply costs for a rotating graduate student, a more senior graduate student and a master's student who are being trained in the laboratory on DIPG and LSD1 inhibition. In addition, Ruby Setara is a new research associate in the Becher lab who is currently applying for a master's program. Training on this project will hopefully excite her about studying this rare and incurable brain cancer in children and she may stay in the Becher lab for her master's program if accepted.

- **How were the results disseminated to communities of interest?**

  A poster and oral presentation at the 2023 ASPET Conference described work related to this project. An abstract will be submitted in November 2023 for the 2024 Annual AACR Conference and will include data generated from the DOD funding.

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

  The in vivo experiments will be prioritized in the next year. We have also begun generating knockdown and knockout lines for LSD1 in the DIPG models. The in vivo LSD1 deletion experiments are ongoing and will be reported in the next reporting period.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

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

     Nothing to report

   - **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:** *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

   - **Changes in approach and reasons for change**

     We will use CUT&RUN in lieu of ChIPseq as the former is more sensitive and requires fewer cells. We received training in CUT&RUN from a collaborating lab (Emily Bernstein).

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

     It has been slow receiving one of the LSD1 inhibitors bomedemstat/IMG-7289 from Merck for clinical studies. We are continuing to work with them despite the delays and expect to receive the compounds for the in vivo testing in year 3.

   - **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

   - **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

6. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
   - **What individuals have worked on the project?**

Name:	Oren Becher
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	ORCID ID: 0000-0003-4016-8492
Nearest person month worked:	2.4
Contribution to Project:	Dr. Becher is overseeing the project.
Funding Support:	

Name:	Joya Chandra
Project Role:	Subcontract PI
Researcher Identifier (e.g. ORCID ID):	ORCID ID: 0000-0003-4016-8492
Nearest person month worked:	2.4
Contribution to Project:	Dr. Chandra is overseeing the project
Funding Support:	

Name:	Samantha Gadd, PhD
Project Role:	*Bioinformatics*
Researcher Identifier (e.g. ORCID ID):	ORCID ID:
Nearest person month worked:	1.2
Contribution to Project:	Performing bioinformatics analysis
Funding Support:	

Name:	Mili Chizhik, BA
Project Role:	Research technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3.5
Contribution to Project:	Technical support: cell culture, tumor induction, and mouse monitoring (Becher lab)
Funding Support:	

Name:	Ruby Setara, BA
Project Role:	Helping with tumor induction and mouse monitoring
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Technical support. Becher lab
Funding Support:	

Name:	Deokhwa Nam
Project Role:	Research scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	performed experiments under Dr. Chandra's mentorship
Funding Support:	

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

None

- **What other organizations were involved as partners?**

  - **Organization Name:** MD Anderson
  - **Location of Organization:** Houston TX
  - **Partner's contribution to the projec**t

    1. **Collaboration** – This award is a collaboration between Mount Sinai (Oren Becher) and MD Anderson (Joya Chandra) as in the award documents.

  - **Organization Name:** Ann & Robert H. Lurie Children's Hospital of Chicago
  - **Location of Organization: Chicago, IL**
  - **Partner's contribution to the project**
    1. **Collaboration** – Sam Gadd, PhD is providing bioinformatics expertise.

Conclusion-

We have made great progress in year 2 as described above, strengthening the likely direct relationship between LSD1 and IL18 in DIPG cells. Interestingly, IL18 is pro-tumorigenic in vivo in the GEMM models while LSD1 inhibition upregulates IL18 levels in vitro and may correlate with antitumor response. For year 3, we are excited for the LSD1 deletion experiments in vivo in both the GEMMs and the xenograft models as well as pharmacological studies in vivo. Lastly, we will continue to study the relationship between LSD1 and IL18. We expect to submit at least one manuscript at the end of the award.