AD_____

Award Number: **W81XWH-18-1-0496**

TITLE: The Role of ATRX/DAXX loss in NF1-associated Solid Malignancies

PRINCIPAL INVESTIGATOR: Fausto J. Rodriguez M.D.

CONTRACTING ORGANIZATION: Johns Hopkins University School of Medicine.

REPORT DATE: Sept 2020

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
Sept 2020	Annual	9/15/2019-9/14/2020

4. TITLE AND SUBTITLE

The Role of ATRX/DAXX loss in NF1-associated Solid Malignancies

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-18-1-0496

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Fausto J. Rodriguez M.D.

E-Mail:frodrig4@jhmi.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Johns Hopkins University
3400 N. Charles St.
Baltimore, MD 21218

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)

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

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

13. SUPPLEMENTARY NOTES

14. ABSTRACT
In this award period, we demonstrated that ATRX knockdown results in ALT-like properties, including increased telomere lengths by PCR based methods and large telomeric foci using telomere specific FISH in MPNST. To more accurately model ATRX loss and ALT in NF1-associated tumors we performed CRISPR mediated TERC knockouts in MPNST cell lines with ATRX loss and are currently characterizing the best clones for in vivo experiments. Our preliminary data demonstrates that the knockdown is successful, that the cells continue to grow, and they lack the expression of senescence markers. We are confident that these models will provide new insights into the role of ATRX loss in the context of NF1-associated tumors and a possible therapeutic role for ATR inhibitors in these difficult to treat tumors.

15. SUBJECT TERMS
NF1, ATRX, DAXX, Alternative lengthening of telomeres, telomeres, glioma, MPNST, pilocytic astrocytoma, diffuse glioma, neurofibroma

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

TABLE OF CONTENTS

1. **INTRODUCTION**

Work from our laboratory has demonstrated that *ATRX* loss and the alternative lengthening of telomeres (ALT) occur frequently in astrocytomas developing in patients with NF1, predominantly adults, and may also develop in a subset of malignant peripheral nerve sheath tumors (MPNST). We have developed several murine and human models to study ATRX in the context of *NF1* loss, and are performing a comprehensive approach to delineate specific phenotypes and functional effects resulting from *ATRX* loss in the context of NF1 tumorigenesis, including effects on telomeres.

2. **KEYWORDS**

NF1, ATRX, DAXX, Alternative lengthening of telomeres, telomeres, glioma, MPNST, pilocytic astrocytoma, diffuse glioma, neurofibroma, neurofibromatosis

3. **ACCOMPLISHMENTS**

- **Major goals of the project**

4. Major Task 1	Months	
Subtask 1 Perform functional experiments using *Atrx* deficient/ *Nf1*$^{+/-}$*Trp53*$^{+/-}$ murine glioma lines.	1-3 (90%)	Dr. Rodriguez
Subtask 2 Perform functional experiments using *ATRX* deficient human NF1- or *BRAF* mut gliomas lines and xenografts.	3-12 (25% HRPO approval very recent and then COVID19 hit)	Dr. Rodriguez
Local IRB/IACUC Approval	3 (100%)	
Local ACURO Approval	3 (100%)	
Major Task 2		
Subtask 1 Establish the optimal oncogene sequence to transform human neural stem cells in the context of NF1 loss.	6-12 (0% HRPO approval very recent and then COVID19 hit)	Dr. Rodriguez
Subtask 2 Study the effects of ATRX loss in glioma initiation using human neural stem cells.	12-18 (0% HRPO approval very recent and then COVID19 hit)	Dr. Rodriguez
Subtask 3: Study phenotypic/telomere alterations resulting from *ATRX* loss in glioma and neural stem cells.	12-18	Drs. Rodriguez and Heaphy
Milestone(s) Achieved: *Obtained functional results and developed xenograft models reflecting ATRX loss during tumor initiation*	18	

Specific Aim 2		
Major Task 3		
Subtask 1 Start in vitro cultures of plexiform neurofibromas and MPNST	6-9 (90%)	Dr. Rodriguez
Subtask 2 Perform *ATRX/DAXX* knockdowns and knockouts in PNST cell lines	9-15 (90%)	Dr. Rodriguez
Milestone(s) Achieved: *Developed PNST cell lines with stable ATRX/DAXX loss for functional experiments and drug screens*	15 (90%)	

Specific Aim 2	**Timeline**	**Site 1**
Major Task 4	Months	
Subtask 1: Develop MPNST xenografts with ATRX/DAXX loss.	15-21 (0% since HRPO approval was recent and COVID; these experiments are now next in line)	Dr. Rodriguez
Subtask 2: Study phenotypic/telomere alterations resulting from *ATRX/DAXX* loss in plexiform neurofibroma and MPNSTs	15-21 (50%)	Drs. Rodriguez and Heaphy
Milestone(s) Achieved: *Measured outcomes secondary to ATRX/DAXX loss in vivo*	25%	
Specific Aim 3		
Major Task 4		
Perform drug treatments using ATR inhibitors using ATRX/DAXX deficient cells.	21-27 (25%)	Dr. Rodriguez

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

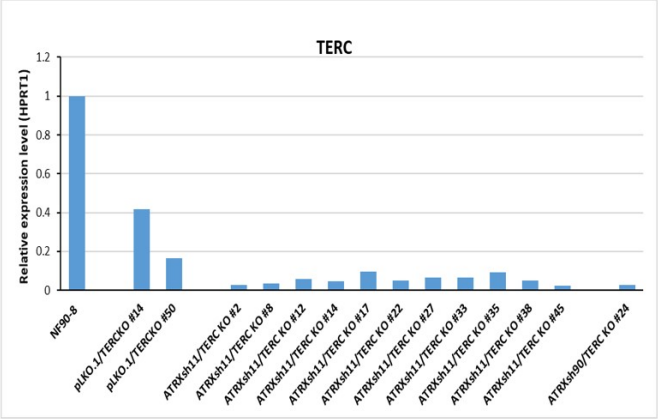
Major Activities and Objectives

1- Created stable ATRX-/TERC- MPNST cell lines for further in vitro and in vivo functional experiments. These experiments are ongoing.

2- Created stable ATRX-/TERC- NF1-deficient high grade glioma cell lines for further in vitro and in vivo functional characterization. These experiments are ongoing

Significant Results

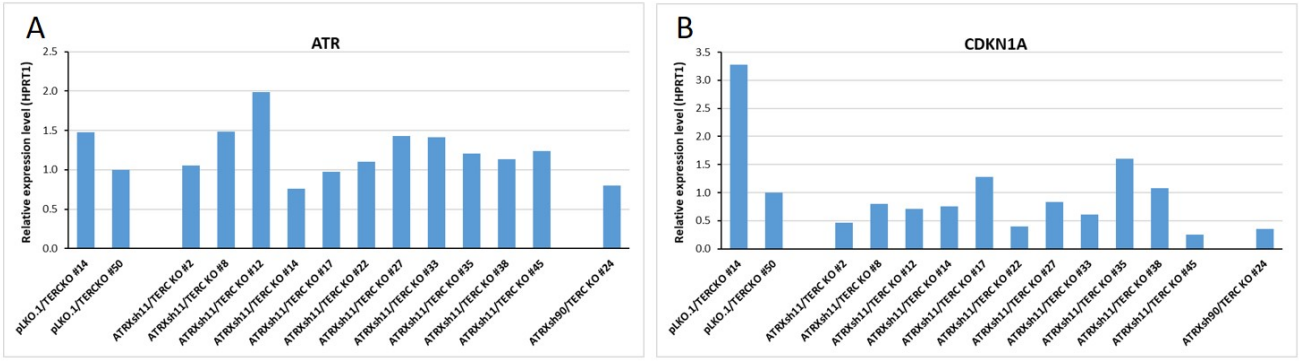
Figure 1. Substantial reduction in *TERC* expression after knockout and *ATRX* knockdown in MPNST cell line NF90-8



<u>Phenotypic/telomere alterations resulting from ATRX/DAXX loss in MPNSTs</u>

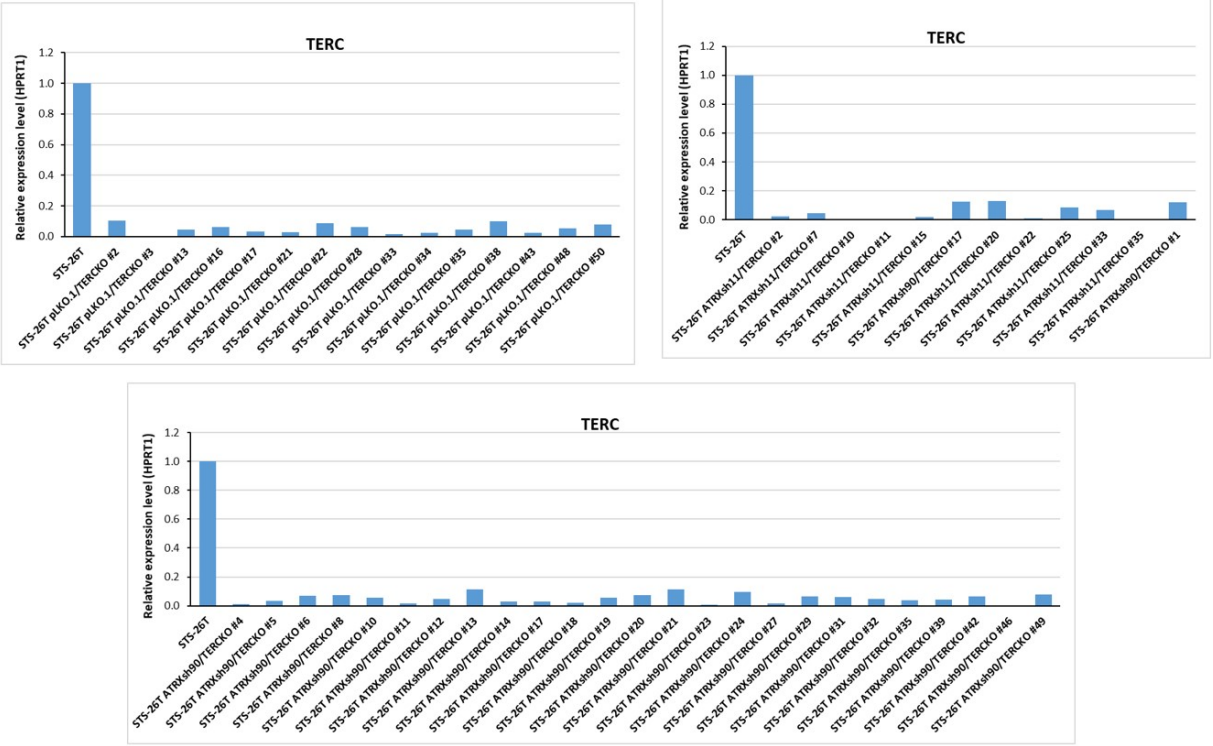
During this part of the project, we focused on selecting the best reagents to test the cooperation of NF1 and ATRX loss on NF1-associated tumorigenesis. We previously used commercially available cell lines to study the effect of ATRX loss in MPNST (ST88-14, NF90-8, STS-26T) and completed a screen for *ATRX, DAXX, NF1 and TERT* promoter alterations. *ATRX* knockdown was efficiently performed in all cell lines, but no significant effects in cell proliferation was noted. However, ALT-like properties were promoted as outlined in our progress report #1. Given that ALT requires an absence of telomerase, we have worked to develop a CRISPR based knockout system to target *TERC* in a background of *ATRX* knockdown to more faithfully model the conditions encountered in cancer. We have developed multiple subclones with the desired combination of ATRX knockdown and *TERC* knockout using cell line NF90-8. TERC expression was substantially low in these clones (**Figure 1**). Given that our subsequent experiments will involve ATR inhibitors as a therapeutic strategy, based on a previously reported vulnerability of ALT positive cells to ATR inhibition (Flynn RL et al. Science 2015;347(6219):273-7.), we found that the clones maintained variable but adequate levels of ATR expression (**Figure 2A**). Additionally, cells continued to grow robustly under culture conditions after multiple passages (~40), with no indication of senescence as measured by a lack of increase in *CDKN1A* mRNA levels compared to controls (**Figure 2B**).

Figure 2. Preserved *ATR* (A) and lack of increase in *CDKN1A* (B) mRNA levels



We also used MPNST cell line STS-26T and successfully were able to knockout *TERC* in a background of *ATRX* loss (**Figure 3**). At the present time we are doing additional phenotypic characterization of these two lines for subsequent xenograft development and testing the effect of ATR inhibition.

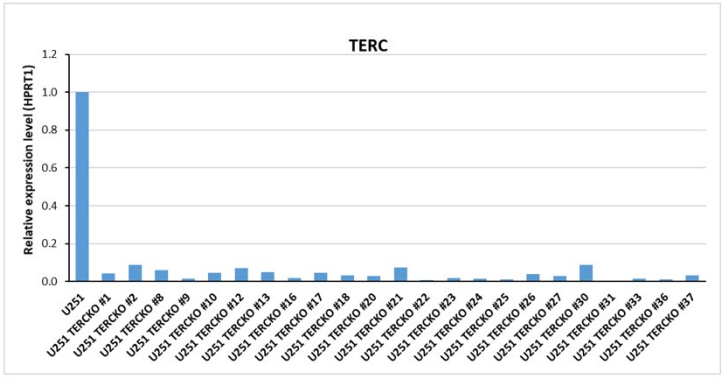
Figure 3. Efficient *TERC* knockout and *ATRX* knockdown in MPNST cell line STS-26T

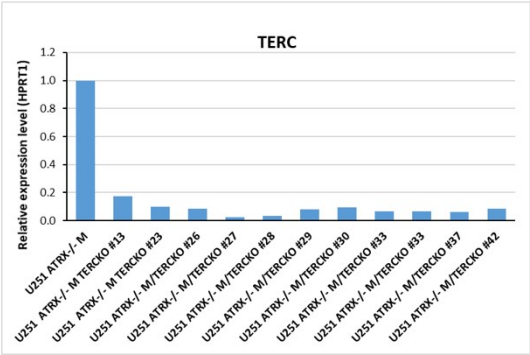


Phenotypic/telomere alterations resulting from ATRX loss in NF1-associated glioma

Given the relevance of ATRX loss and the ALT phenotype to NF1-associated glioma we have also studied the phenotypic alterations resulting of combined *ATRX* and *NF1* loss. In previous unpublished data, we demonstrated the development of ALT-like properties in *Nf1*$^{+/-}$*Trp53*$^{+/-}$ murine glioma lines after Atrx knockdown and pharmacologic telomerase inhibition. Subsequently we performed *ATRX* knockdown in a NF1-associated low grade glioma line, but subsequent experiments were not possible given induction of senescence. Therefore, we chose to use two NF1-deficient high grade glioma cell lines (U251 and SF188) for further experiments. *ATRX* knockout was previously reported in these two cell lines by our co-investigators leading to ALT in U251 but not SF188 (Brosnan-Cashman J et al. PLOS ONE 13(9): e0204159). We used these ATRX deficient cells and performed *TERC* knockouts to more faithfully model the ALT state. We confirmed the substantial decrease in *TERC* levels in multiple subclones (**Figure 4 and 5**). As with the MPNST lines, we are doing additional phenotypic characterization, with the aim to test ATR inhibition *in vitro* and *in vivo.*

Figure 4. Efficient *TERC* and *ATRX* knockout in *NF1*-deficient glioma cell line U251





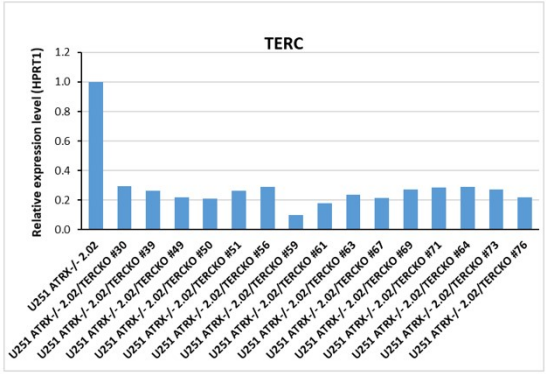
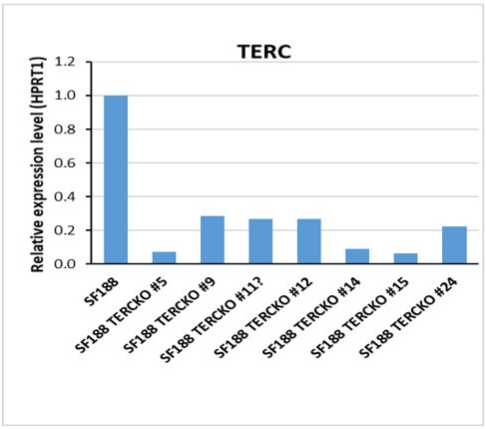


Figure 5. Substantial reduction in *TERC* expression after knockout in *NF1*-deficient glioma cell line SF188



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

Sarra Belakhoua is a senior medical student from the Faculty of Medicine of Tunis, Tunisia, who completed a two month research rotation as an external visitor under Fausto Rodriguez supervision from January to March of 2020. During her time in our lab she became proficient in the application of immunohistochemistry and analysis of publically available genomic databases of brain tumors. During her time at Hopkins she co-authored one case report and first-authored a comprehensive review on the pathology of peripheral nerve tumors. Her research project focused on the status of RECQL4 expression and ALT in NF1-deficient neoplasms, a side project that developed from data arising from our overall grant. She presented her findings in our laboratory meetings and at the yearly Johns Hopkins Department of Pathology Young investigators Day, which was well received. We anticipate publishing a manuscript sometime this year. She is currently in the application process for pathology residency training in the United States.

Fausto Rodriguez (PI). The PI has reinforced his collaborations with the Brain Research Institute, Niigata University, Japan in the context of a grant focusing on the role of autophagy in NF1-associated gliomas. He has applied for a collaborative international fellowship to the *Japan Society for the Promotion of Science* to continue this collaboration with a focus on NF1-associated gliomas. Dr. Rodriguez has also presented the data originating in this grant in numerous national and international venues, including a special lecture delivered at the annual meeting at the Spanish Society of Neurology, Seville, Spain, and an oral presentation at the American Association of Neuropathologists. He was also invited to participate and present this data at the *Neurofibromatosis type 1 and RAS symposium: Consideration for Gene Therapy*, organized by the Frederick National Lab Advanced Technology Research Facility, Frederick, MD to brainstorm about possible research and clinical trials in the field. More recently, Dr. Rodriguez has been promoted to Professor of pathology, oncology and an ophthalmology at the Johns Hopkins University School of Medicine. His contribution to the pathology of neurofibromatosis type 1 tumors, and the ongoing work on this grant, were important highlights on his promotion paperwork.

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

  Nothing to report.

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

The most important next steps is to extend our initial functional experiments to more relevant models of NF1 and ATRX loss, particularly patient derived glioma and MPNST cells lines in the context of murine xenografts. We are proceeding to develop in vitro models of ATRX/NF1 loss in the context of TERT inactivation (a more accurate combination reflective of human disease). We are finalizing our selection of the best clones so we can start studying the effects of specific drugs in vitro (ATR inhibitors) and create the appropriate xenografts to study in vivo. Our laboratory is currently active after the COVID-19 lockdown and the HRPO and AUCU protocol approvals are all in place, so we anticipate substantial progress in our experimental efforts in this upcoming year.

5. **IMPACT**

   - **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

6. **CHANGES/PROBLEMS**

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

     Nothing to report

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

We encountered two problems that slowed somewhat the progress of our work during this past year:
1-There was a significant delay from ACURO and HRPO to approve the standard approaches we propose to study glioma and MPNST cell lines in vitro and in vivo (xenografts). We were told that there were staff shortages at the time. We received ACURO approval just in the middle of the prior reporting process, and HRPO more recently.

2-COVID-19 pandemic. We were able to adapt to many of the closures and delays related to the pandemic at multiple levels. Key effects on our lab included work at

significantly reduced capacity given institutional restrictions and slow deliveries of necessary reagents. We were therefore unable to start our plans for in vivo experiments, but will be continuing with them shortly.

- **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

7. **PRODUCTS**

   - **Publications, conference papers, and presentations**
     - **Journal publications.**

Heaphy C, Bi WL, Coy S, Davis C, Gallia G, Santagata S, **Rodriguez FJ**. Telomere length alterations and ATRX/DAXX loss in pituitary adenomas. *Mod Pathol* 2020;33:1475-1481

Arnold A, Imada EL, Zhang L, Edward DP, Marchionni L**, Rodriguez FJ**. Differential gene methylation and expression of *HOX* transcription factor family in orbitofacial neurofibroma. Acta Neuropathol Comm 2020;8(1):62

Pollard K, Banerjee J, Doan X, Wang J, Guo X, Allaway R, Langmead S, Slobogean B, Meyer CF, Loeb D, Morris C, Belzberg A, Blakeley J, **Rodriguez F**, Guinney J, Gosline S, Pratilas C. A clinically and genomically annotated nerve sheath tumor biospecimen repository. *Scientific Data* 2020 Jun 19;7(1):184.

Nix JS, Yuan M, Imada EL, Ames H, Marchionni L, Gutmann DH, **Rodriguez FJ**. Global microRNA Profiling Identified miR-10b-5p as a Regulator of Neurofibromatosis 1 (NF1)-glioma Migration. *Neuropathol Appl Neurobiol* (advanced online publication)

Belakhoua S, **Rodriguez FJ**. Diagnostic pathology of tumors of peripheral nerve. *Neurosurg* (in press)

Books or other non-periodical, one-time publications

Contributing author. Diagnostic Pathology: Familial Cancer Syndromes. Second Edition. Elsevier. 2020

Perry A, Reuss DE, **Rodriguez F**. Neurofibroma. WHO Classification of Tumours of the Soft Tissue and Bone. Fourth Edition. IARC Press. 2020.

.

Other publications, conference papers, and presentations.

9/19 **Rodriguez FJ**, Graham MK, Brosnan-Cashman JA, Barber JR, Davis C, M., Vizcaino MA, Palsgrove DM, Giannini C, Pekmezci M, Dahiya S, Gokden M, Noë M, Wood LD, Pratilas CA, MorrisC, Belzberg A, Blakeley J. **Heaphy CM**. Telomere alterations in neurofibromatosis type 1-associated solid tumors. NF conference abstract book 2019, p.124 (Poster presentation at the annual Children's Foundation (NF conference), San Francisco, CA

10/19 Speaker, Neurofibromatosis type 1 and RAS symposium: Consideration for Gene Therapy, *Role of ATRX and alternative lengthening of telomeres in neurofibromatosis type 1-associated solid malignancies*, NIH/Frederick, MD

11/19 Pathcast Videos. *Tumors of Peripheral Nerve* 11/26/2019 (https://www.facebook.com/pathCast/videos/2536590846429556/)

11/19 Speaker, LXXI Meeting of the Spanish Society of Neurology, *Advances in the pathology of NF1-associated solid tumors*, Seville, Spain

6/20 Yuan M, Reilly K, Pratilas C, Heaphy C, **Rodriguez FJ**. Functional characterization of ATRX loss in NF1-associated glioma and MPNST. *J Neuropathol Exp Neurol* 2020; 79: 654-716 (Oral presentation at the annual meeting of the American Association of Neuropathologists, June 2020)

7/20 Speaker, *Brain tumors in NF1 patients*, DASA Pathology Weekly Conference, Brasil.

7/20 Speaker, Department of Pathology of Pathology and Laboratory Medicine, University of California Los Angeles, Advances in the Pathology of Neurofibromatosis type 1-associated Solid Tumors, Zoom Meeting

- **Website(s) or other Internet site(s)**
  Nothing to report
- **Technologies or techniques**
  Nothing to report
- **Inventions, patent applications, and/or licenses**
  Nothing to report

- **Other Products**
  Nothing to report

8. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

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

     Fausto Rodriguez (PI): no change.

     Ming Yuan: no change.

     Christopher Heaphy: Moved to assume a faculty positon at Boston University. Dr. Alan Meeker, who worked closely with him in the same laboratory, transitioned to his role, including same effort and responsibilities as outlined below:

Name:	*Alan Meeker*
Project Role:	*Faculty Co-investigator*
Researcher Identifier (e.g. ORCID ID):	NA
Nearest person month worked:	1.20
Contribution to Project:	*Dr. Meeker has participated in group meetings for the project, provided technical and conceptual advice and assisted in the evaluation of telomere alterations in our experimental models.*
Funding Support:	*NA*

Name:	*Sarra Belakhoua*
Project Role:	*Visiting Medical Student*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	1
Contribution to Project:	*Ms. Belahkoua performed immunohistochemical studies, analyzed data and wrote a review manuscript of the pathology of peripheral nerve sheath tumors*
Funding Support:	*NA*

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

  Nothing to report

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

  Nothing to report

9. **SPECIAL REPORTING REQUIREMENTS**

   Nothing to report

10. **APPENDICES:**

    Nothing to report.