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TITLE: Testing the Role of p21 Activated Kinases in Schwannoma Formation Using a Novel Genetically Engineered Murine Model that Closely Phenocopies Human NF2 Disease

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14. ABSTRACT

Neurofibromatosis type 2 (NF2) is an autosomal dominant genetic disease characterized by benign schwannomas that grow on the cranial and spinal nerves. While technically benign, the tumors are nonetheless progressive and relentless, usually resulting in death before age fifty from inoperable intracranial masses. To date, surgery remains the only effective therapy for these lesions, though this therapy is frequently associated with major morbidities, including loss of hearing. In this award we proposed to evaluate Group A Paks (Pak1 and Pak2) as a therapeutic target in NF2 by intercrossing Group A PAK deficient mice with our NF2 mouse model (*PostnCre;Nf2$^{flox/flox}$*). Briefly, we hypothesized that the knockdown of Group A Paks in Nf2 deficient mice will rescue or reduce tumorigenesis. In **Specific Aim 1**, we proposed to characterize Pak's signaling influence on NF2 *in vivo* by assessing hearing thresholds, survival rate, tumor formation and kinome analysis. In **Specific Aim 2**, we proposed to test Pak inhibitors in our NF2 tumor model in order to evaluate whether the inhibition of Pak rescues merlin function to wild-type levels by analyzing readouts generated from tumor growth and auditory brainstem response (ABR).

15. SUBJECT TERMS
NF2, Pak1, Pak2, PID, ABR, Pak inhibitors

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INTRODUCTION

Neurofibromatosis type 2 (NF2) is an autosomal dominant genetic disease characterized by benign schwannomas that grow on the cranial and spinal nerves. While technically benign, the tumors are nonetheless progressive and relentless, usually resulting in death before age fifty from inoperable intracranial masses. To date, surgery remains the only effective therapy for these lesions, though this therapy is frequently associated with major morbidities, including loss of hearing. In this award we proposed to evaluate Group A Paks (Pak1 and Pak2) as a therapeutic target in NF2 by intercrossing Group A PAK deficient mice with our NF2 mouse model (*PostnCre;Nf2$^{flox/flox}$*). Briefly, we hypothesized that the knockdown of Group A Paks in Nf2 deficient mice will rescue or reduce tumorigenesis. In **Specific Aim 1**, we proposed to characterize Pak's signaling influence on NF2 *in vivo* by assessing hearing thresholds, survival rate, tumor formation and kinome analysis. In **Specific Aim 2**, we proposed to test Pak inhibitors in our NF2 tumor model in order to evaluate whether the inhibition of Pak rescues merlin function to wild-type levels by analyzing readouts generated from tumor growth and auditory brainstem response (ABR).

KEYWORDS: NF2, Pak1, Pak2, PID, ABR, Pak inhibitors

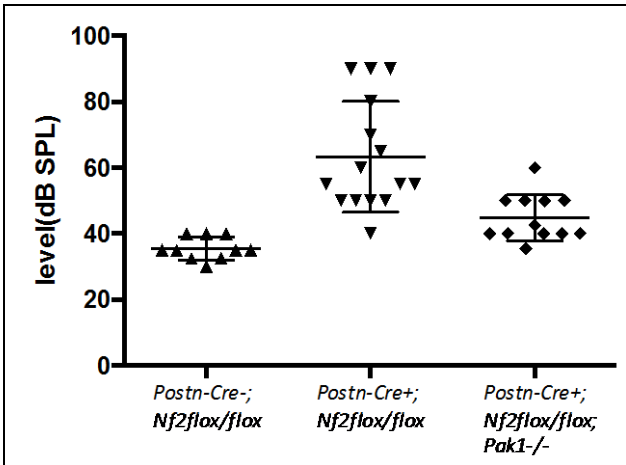

Figure 1. ABR study quantitating from 9-month- old Cre-negative control,Postn-Cre+; Nf2$^{flox/flox}$, Postn-Cre+; and Postn-Cre+; Nf2$^{flox/flox}$;Pak1-/- mice, respectively. Each dot represents the average in left and right threshold of an individual ($P < 0.0001$).

ACCOMPLISHMENTS

(A) Major Goals of the Project

The major goal of this research project was to genetically and pharmacologically test the requirement of Group A PAK signaling in Nf2 deficient schwannoma genesis. We would accomplish this goal by (1) using our genetically intercrossed Pak1, Pak2 and a dominant negative Group A PAK transgene with our *PostnCre;Nf2$^{flox/flox}$* mouse schwannoma model in order to generate functional (ABR testing) and histological readouts. In turn these readouts will allow us to utilize a genetic approach to determine if indeed PAK signaling is essential to the development of *Nf2*-deficient schwannomas; (2) using histological and ABR readouts, we will treat *PostnCre;Nf2$^{flox/flox}$* mice with three different pharmacologic PAK inhibitors to determine if targeted PAK inhibition in a preclinical model of schwannoma genesis rescues tumor development.

(B) Accomplishments under these Goals

In **Specific Aim 1a.1**, we proposed to characterize the role of individual Pak isoforms (Pak1 and Pak2) by intercrossing these mice with our NF2 mouse model, *Postn-Cre; Nf2$^{flox/flox}$* to generate the following cohorts of mice; 15 *Postn-Cre-; Nf2$^{flox/flox}$; Pak1$^{+/+}$* mice (Control), 15 *Postn-Cre+; Nf2$^{flox/flox}$; Pak1$^{+/+}$* mice (NF2-KO), 15 *Postn-Cre-; Nf2$^{flox/flox}$; Pak1$^{-/-}$* mice (PAK1-KO), and 15 *Postn-Cre+; Nf2$^{flox/flox}$; Pak1$^{-/-}$* (NF2/PAK1-DKO). These cohorts of mice were utilized in order to assess hearing loss via auditory brainstem response (ABR) testing. Preliminary data included in the initial grant proposal indicated that our *Postn-Cre+; Nf2$^{flox/flox}$* mice showed by eight months of age, complete hearing loss, as compared to control mice, *Postn-Cre-; Nf2$^{flox/flox}$*. Functionally, the mean hearing threshold of nearly 60 dB in *Postn-Cre+; Nf2$^{flox/flox}$* mice at the age of 10 months is equivalent, as a human analog, of the inability to hear in a normal conversation, thus indicating severe disruption of an affected individual's life. In the analysis of 9 to 10 month old *Postn-Cre+; Nf2$^{flox/flox}$;Pak1$^{-/-}$* mice we observed that these mice showed improved hearing as compared 9 to 10 month old Nf2 deficient mice (*Postn-Cre+; Nf2$^{flox/flox}$* mice), as shown in **Fig. 1**. Control mice (*Postn-Cre-; Nf2$^{flox/flox}$; Pak1$^{+/+}$*) showed no hearing loss at 9 to 10 months of age. We ended the ABR testing at 10 months of age for the *Pak*1 experimental cohorts and their *Nf2* deficient litter mates enrolled into the study as the *Nf2* deficient mice was having dramatic hearing loss, experiencing significant pain due to tumor burden and their survival rate was rapidly decreasing after 10 months of age.

Therefore, after consulting with our colleagues, we decided to end this study for humane and ethnical reasons at 10months of age for all enrolled Pak1 deficient and their Nf2 deficient littermates enrolled into the study. From the data collected at 10 months of age, we can conclude that upon genetically knocking out Pak1 in Nf2 deficient mice improves hearing loss.

In the analysis of 4 to 6 months old *Postn-Cre+; $Nf2^{flox/flox}$;$Pak2^{-/-}$ we have observed that these mice showed no changes in hearing thresholds as compared to Postn-Cre+; $Nf2^{flox/flox}$* mice cohorts (data not shown). However, as discussed below, there are challenges in long term evaluation of this strain due to the formation of new tumors.

In **Specific Aim 1a.2**, we proposed to characterize the role of individual Pak isoforms (Pak1 and Pak2) in the NF2 mouse model by assessing tumor formation. Our preliminary data indicate that genetic disruption of Pak 1 or Pak 2 is not completely sufficient to inhibit tumor initiation (**Figs. 2 and 3**). However, there is a strong but not statistically significant suggestion that genetic disruption of Pak1 is resulting in a reduction in dorsal root ganglia size as illustrated in **Fig. 2**. Though we need to evaluate additional specimens, the histology of the dorsal root ganglia (DRG) of mice containing a disruption of the Pak1 allele appears more benign than the *Postn-Cre+; $Nf2^{flox/flox}$;$Pak2^{flox/flox}$* and the *Postn-Cre+; $Nf2^{flox/flox}$*. However, more studies will be required to verify this observation.

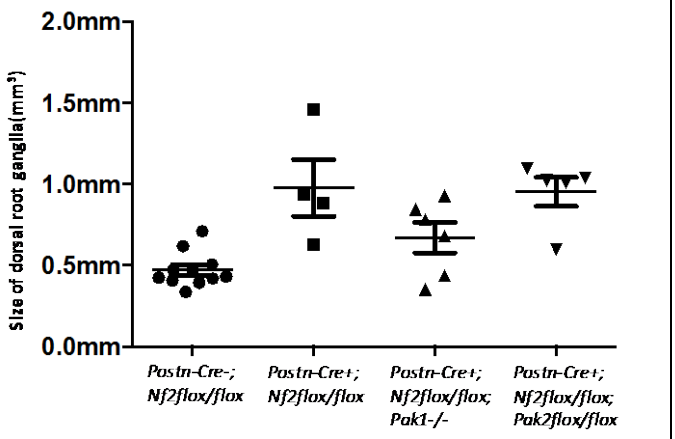

Figure 2. Mean size of dorsal root ganglia (DRG) from 10-month- old Cre-negative control,Postn-Cre+; $Nf2^{flox/flox}$, Postn-Cre+; $Nf2^{flox/flox}$;$Pak2^{flox/flox}$ and Postn-Cre+; $Nf2^{flox/flox}$;Pak1-/- mice, respectively. Each dot represents the size of an individual DRG. The line indicates the mean size of all DRG ($P < 0.1212$).

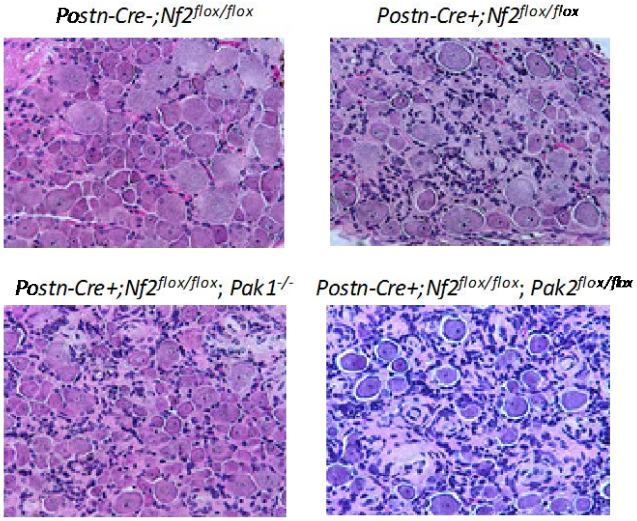

Figure 3. H&E stain of the DRG from 10-monthy old Cre-negative control, *Postn-Cre-;$Nf2^{flox/flox}$*, *Postn-Cre+;$Nf2^{flox/flox}$; $Pak1^{-/-}$*, and *Postn-Cre+;$Nf2^{flox/flox}$; $Pak2^{flox/flox}$* mice, respectively. *Postn-Cre+;$Nf2^{flox/flox}$; $Pak1^{-/-}$* mice slowly develop schwannomas of the DRG and spinal nerved compared to *Postn-Cre+;$Nf2^{flox/flox}$*.

Diffuse spinal nerve hyperplasia and pseudo-onion bulb formation of proliferating schwann cells are observed in the nerve of a *Postn-Cre+;$Nf2^{flox/flox}$;$Pak1^{-/-}$* mice, however, the frequency is lower. Original magnification x40.

In **Specific Aim 1a.3**, we proposed to characterize the role of individual Pak isoforms (Pak1 and Pak2) in our NF2 mouse model, *Postn-Cre; $Nf2^{flox/flox}$*, by assessing survival rate. Preliminary data included in the initial grant proposal indicated that our *Postn-Cre+; $Nf2^{flox/flox}$* mice showed by eight months of age, a decrease in survival as compared to control mice, *Postn-Cre-; $Nf2^{flox/flox}$*. By 20 months of age, roughly less than 40% of the Nf2/Pak1 deficient mouse cohort survival as compared to roughly 15% NF2 deficient cohort survival (**Fig. 4**). This data indicates that genetically knocking out *Pak1* in *Nf2* deficient mice rescues overall survival as compared to Nf2

deficient cohorts. No significant changes of survival rate was observed in the *Nf2$^{flox/flox}$;Pak1+/+, Postn-Cre-(WT)* cohort of mice.

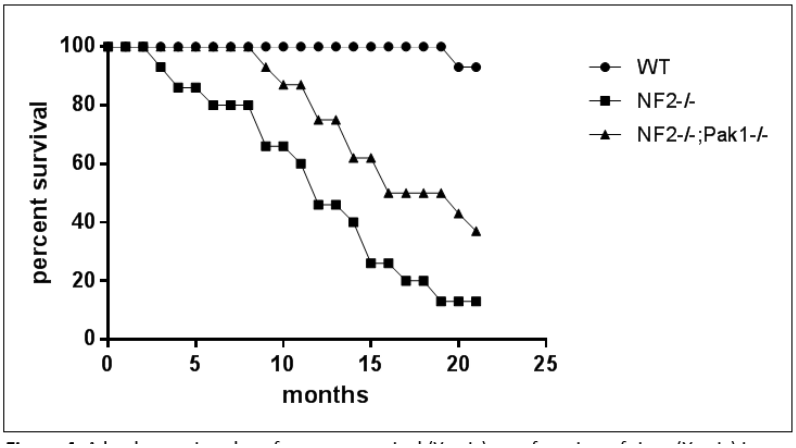

Figure 4. A Kaplan-Meier plot of percent survival (Y-axis) as a function of time (X-axis) is shown. Groups are *Cre*-negative control (n=15), *Postn-Cre+;Nf2$^{flox/flox}$* (n=15), and *Postn-Cre+;Nf2$^{flox/flox}$;Pak1$^{-/-}$* (n=16) mice, respectively.

Currently, we have cohorts of *Nf2$^{flox/flox}$* and *Pak2*$^{flox/flox}$ enrolled in survival studies and during the no cost extension period we will continue to observe these cohorts. The enrolled cohorts are 15 *Postn-Cre-; Nf2$^{flox/flox}$; Pak2$^{flox/flox}$* mice (Control) and 15 *Postn-Cre+; Nf2$^{flox/flox}$; Pak2$^{flox/flox}$* (NF2/PAK2-DKO). Additionally, upon intercrossing the *Postn-Cre-; Nf2$^{flox/flox}$ with Pak2$^{flox/flox}$ mice in order to generate the Nf2$^{flox/flox}$; Pak2$^{flox/flox}$* mice (PAK2-KO), we observed by 2 months of age, these mice developed hind limb paralysis and had a body score of 1. Therefore, upon consultation with our animal facilities veterinarian and colleagues, we decided to end the survival studies for the genetically modified single knockout cohorts *Postn-Cre+; Nf2$^{+/+}$; Pak2$^{flox/flox}$* mice (Pak2-KO) and *Postn-Cre+; Nf2$^{Flox/Flox}$; Pak2$^{+/+}$* mice (Nf2-KO).

In Specific Aim 1B, we had planned to intercrossed the planned cross of *Postn-Cre*; *Nf2$^{flox/flox}$* mice with ROSA26-LSL-PID mice and analyze tumor growth and hearing loss (Months 6-30). However, given the results of the Pak1 and Pak2 intercrosses, we concluded that crosses with the ROSA26-LSL-PID mouse were not necessary.

In **Specific Aim 1C** we proposed to assess signaling activity in tumors from the following cohorts of mice: *Postn-Cre; Nf2$^{flox/flox}$* Pak1$^{-/-}$, and *Postn-Cre; Nf2$^{flox/flox}$* Pak2$^{flox/flox}$, and ROSA26-LSL-PID mouse models. We carried out an analysis of signaling from the first of these crosses, *i.e.*, *Postn-Cre; Nf2$^{flox/flox}$* vs *Postn-Cre; Nf2$^{flox/flox}$*; Pak1$^{-/-}$ mice. Tumors were collected post ABR testing (performed in Aim1a.1) starting at 10 months of age, and at 12 months and at 14 months.

We pooled protein lysates derived from three schwannomas from *Postn-Cre*;*Nf2$^{flox/flox}$*;*Pak1$^{+/+}$ and* three *Postn-Cre*;*Nf2$^{flox/flox}$*;*Pak1$^{-/-}$* mice, respectively, and subjected these lysates to western blot analysis using a variety of antibodies, as shown in **Fig. 5**. Our data show that loss of Pak1 resulted in reductions in phosphorylation of a known Pak1 substrate, Mek1 S298, as well as partial loss of P-Erk, p38 signals, and p-β-catenin signals.

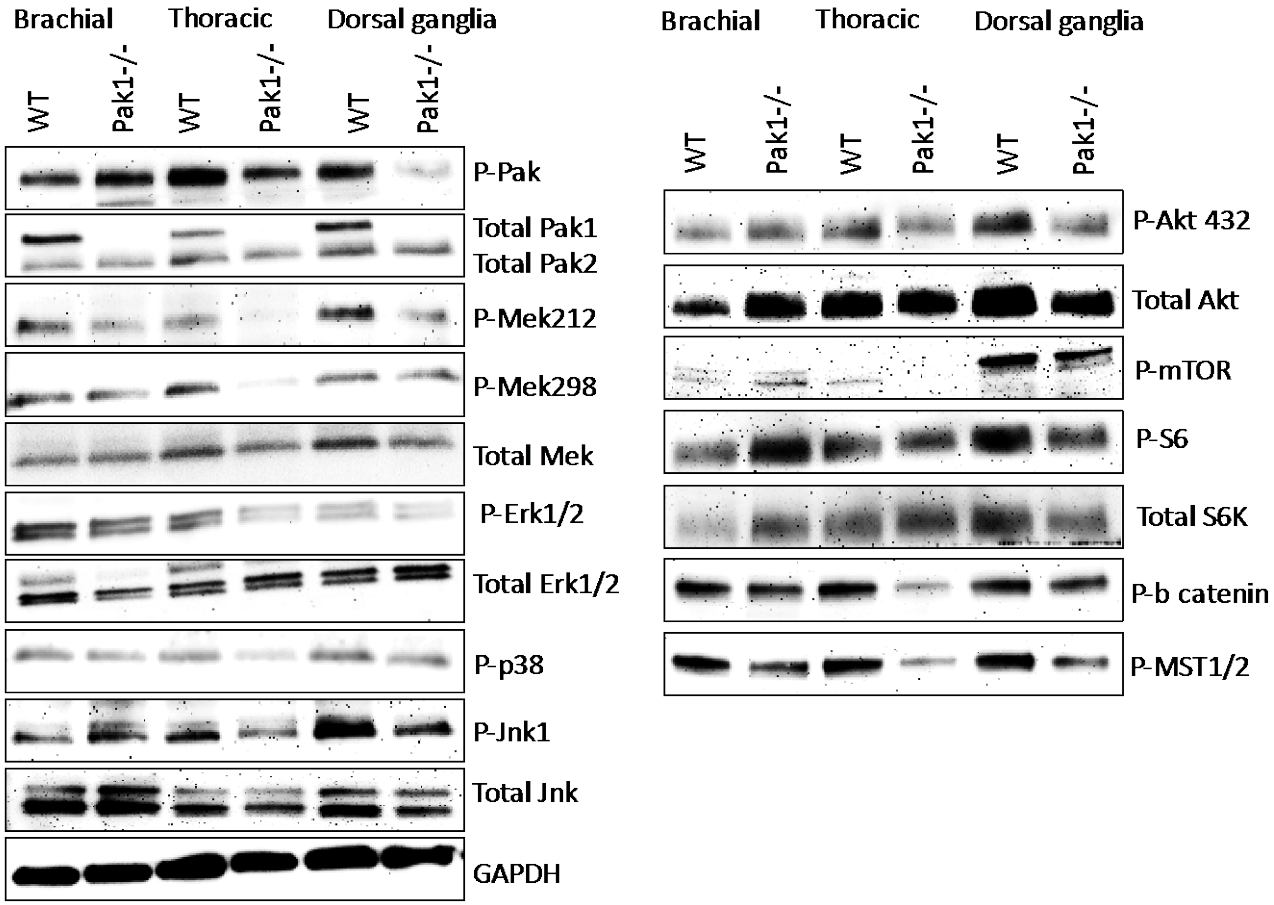

Figure 5. Western blot analysis of tumors from *Postn-Cre+;Nf2$^{flox/flox}$ vs. Postn-Cre+;Nf2$^{flox/flox}$;Pak1$^{-/-}$* mice.

In **Specific Aim 2** we proposed to test small molecule Pak inhibitors on our *Postn-Cre; Nf2$^{flox/flox}$* mouse model. During this reporting period, we completed these studies using FRAX-1036, a specific inhibitor of group A Paks. The compound was well tolerated in our murine Schwannoma model at the 30mg/kg/d dose and demonstrated robust inhibition of Pak phosphorylation *in vivo* (**Fig. 6B and 6C)**.

Despite inhibition of Pak phosphorylation, tumor size was not significantly decreased in FRAX-1036 treated mice. ABR analysis revealed a modest decrease in hearing threshold after 12 weeks of treatment versus vehicle control treated mice, but the decrease was not statistically significant (**Fig. 7**). Based on ongoing work (reviewed in Radu et al., *Nat Rev Cancer*. 2014, **14**:13), we believe these results reflect opposing roles for the two main Pak isoforms, Pak1 and Pak2. The genetic experiments clearly show a role for Pak1, but not Pak2, in NF2 tumor growth, whereas the small molecule inhibitor FRAX-1036, which blocks Pak1 as well as Pak2, was ineffective (**Fig. 7**). Accumulating evidence from our labs and others show that Pak2 plays a tumor suppressive function, in opposition to Pak1, in many tissue types. If so, a Pak1 isoform specific small molecule inhibitor may be required in NF2. A prototype of such an inhibitor has been described by Novartis (Karpov *et a*l. *ACS Med. Chem. Lett.*, 2015, **6**:776)

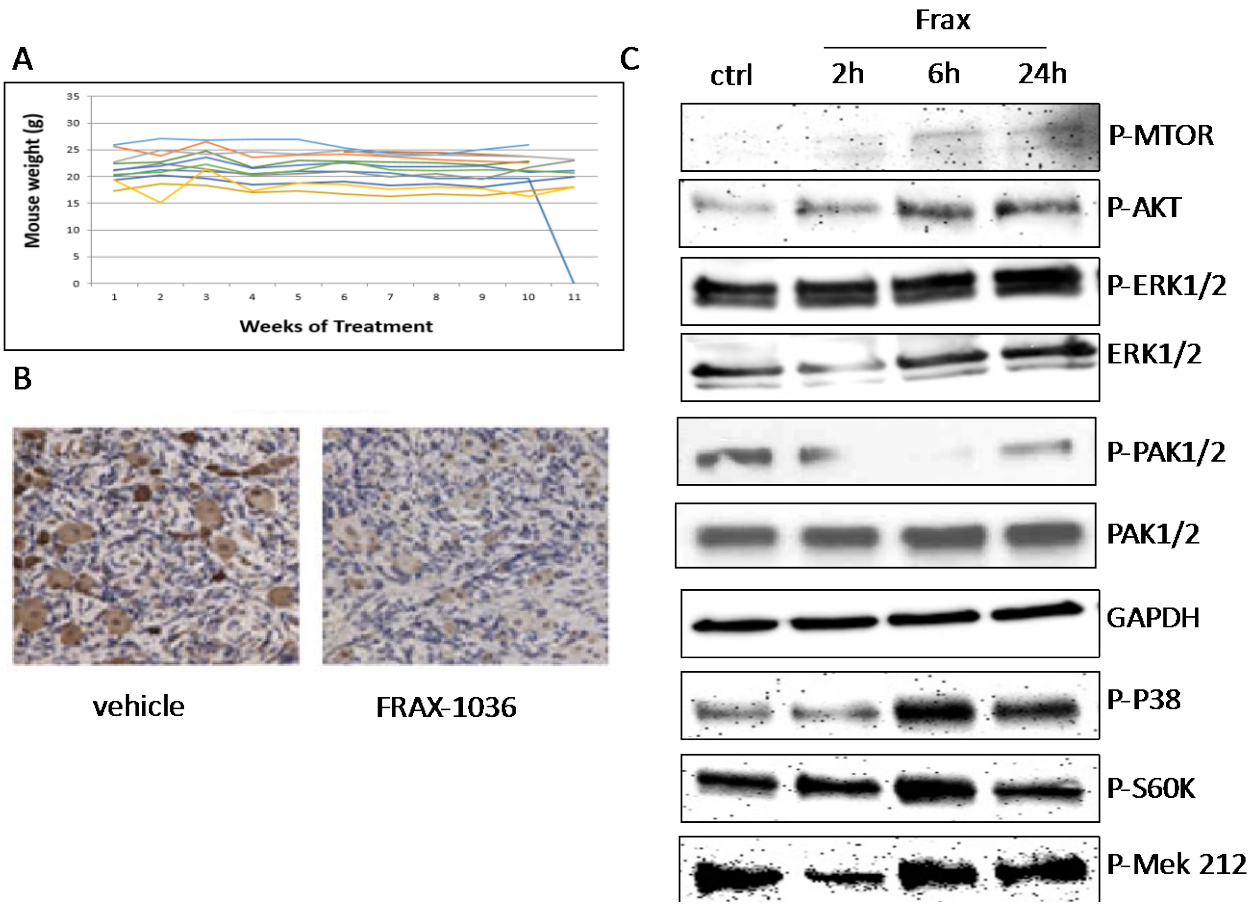

Figure 6. (A) Mouse weights from FRAX-1036 treated mice. (B) IHC staining of DRGs using anti-phospho-Pak. (C) Immunoblot analysis post dosage with vehicle or FRAX-1036.

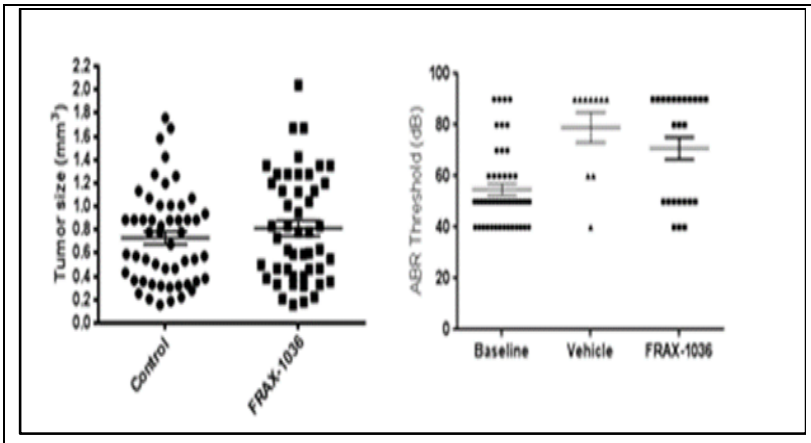

Figure 7. (A) DRG size from vehicle and FRAX-1036-treated mice. Each dot represents an individual DRG. (B) Baseline and post-treatment ABR thresholds from vehicle and FRAX-1036-treated mice. Each dot represents an individual ear measurement.

(C) Training and Professional Development Opportunities

Nothing to Report

(D) Disseminated Results to Communities of Interest

Dr. Clapp has been invited to present this work at the upcoming NF/CTF conference in Paris, France, November 2-6, 2018. In addition, the two PIs have a manuscript in preparation.

IMPACT

Nothing to Report

CHANGES/PROBLEMS

Aim 1a.1: We ended the ABR testing at 10 months of age for the *Pak*1 experimental cohorts and their *Nf2* deficient litter mates enrolled into the study as the *Nf2* deficient mice was having dramatic hearing loss, experiencing significant pain due to tumor burden and their survival rate was rapidly decreasing after 10 months of age. Therefore, after consulting with our colleagues, we decided to end this study for humane and ethnical reasons at 10 months of age for all enrolled Pak1 deficient and their Nf2 deficient littermates enrolled into the study. From the data collected at 10 months of age, we can conclude that upon genetically knocking out Pak1 in Nf2 deficient mice improves hearing loss.

Aim 1a.3: Upon intercrossing the *Postn-Cre-; $Nf2^{flox/flox}$ with $Pak2^{flox/flox}$ mice in order to generate the $Nf2^{flox/flox}$; $Pak2^{flox/flox}$* mice (PAK2-KO), we observed by 2 months of age, these mice developed hind limb paralysis and had a body score of 1. Therefore, upon consultation with our animal facilities veterinarian and colleagues, we decided to end the survival studies for the genetically modified single knockout cohorts *Postn-Cre+; $Nf2^{+/+}$; $Pak2^{flox/flox}$*.

PRODUCTS

Nothing to Report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	***J. Chernoff, M.D., Ph.D.***
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-4803-7836
Nearest person month worked:	*1 calendar month*
Contribution to Project:	*Overall administration and guidance of research; Management and training of personnel*
Funding Support:	*N/A*
Name:	***H-Y. Chow, Ph.D.***
Project Role:	Research Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6 calendar months*
Contribution to Project:	*Kinome screening using MIBs; Examining tumor tissues by IHC and immunoblot*
Funding Support:	*N/A*
Name:	***D. Wade Clapp, MD***
Project Role:	*Subcontract PI*

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*0.21*
Contribution to Project:	*Oversee the laboratory infrastructure as well as the experimental studies performed in his laboratory*
Funding Support:	*N/A*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Not applicable.
What other organizations were involved as partners?

Organization Name: Indiana University, School of Medicine
Location of Organization: Indianapolis, IN
Partner's contribution to the project: Dr. Chernoff developed the *Pak1*$^{-/-}$ and *Pak2*$^{flox/flox}$ mice, while Dr. Clapp developed the *Postn-Cre-;Nf2*$^{flox/flox}$ mouse model. Mouse crosses, tumor analysis, and hearing tests were done in Dr. Clapp's laboratory, whereas signaling analyses were done in Dr. Chernoff's laboratory.

SPECIAL REPORT REQUIREMENTS
Not applicable.

APPENDICES
Not applicable.