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Award Number: W81XWH-11-1-0143

TITLE: SNF5 mutation leads to intractable pain in schwannomatosis patients

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REPORT DATE: July 2013

TYPE OF REPORT: Final

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
July 2013	Final	15 June 2011 – 14 June 2013

4. TITLE AND SUBTITLE

SNF5 mutation leads to intractable pain in schwannomatosis patients

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0143

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)

Oregon Health and Sciences University
Portland, OR 97239

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

Ablation of snf5, in vivo, results in an increase in the number and type of sensory neurons that express the capsaicin receptor, TRPV1. Using a culture system, we have determined that soluble factor is released by snf5-/- Schwann cells that acts on sensory neurons to induce the expression of TRPV1. This factor is greater than 10K molecular weight and does not affect neuron survival. The increase in the immunoreactivity of TRPV1 is correlated with an increase in the expression of functional capsaicin-sensitive ion fluxes.

15. SUBJECT TERMS
Capsaicin receptor expression, conditioned medium factor

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
U	U	U	UU	11	**19b. TELEPHONE NUMBER** *(include area code)*

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Introduction.

Schwannomatosis is a devastating disease that does not affect longevity but has profound effects on the patient's quality of life due to the presence of intractable pain. The cause of this pain is not known. Our hypothesis is that mutations in the SNF5 gene in Schwann cells and ganglionic satellite cells leads to enhanced pain sensitivity in peripheral sensory neurons (see Campana et al, 2007). Mutations in the human SNF5 gene are linked to schwannomatosis (Hulsebos et al., 2007; Boyd et al.,2008; Hadfield et al., 2008; Sestini et al., 2008; Patil et al., 2008). Mice homozygous for snf5 deletion are embryonic lethal while heterozygotes develop rhabdoid tumors and other malignancies (Roberts et al., 2000; Klochendler et al., 2000). We are using a tomoxifen inducible Cre-mediated recombination system driven by a mouse proteolipid protein-1 (*Plp1*) promoter (Plp1-cre/ESR1;Jackson Labs) to target the KO to Schwann and satellite cells. When this mouse is crossed with a floxed snf5 mouse, gene activity is reduced by >80% in the peripheral nervous system. A description of our aims for this project and our preliminary data follows.

Project Proposed Aims and Results.

The first part of this report of results is essentially identical to our yearly progress report **(posted 2/12/2013).** To this belated annual report we add newly obtained data indicating a induction of capsaicin sensitivity by Schwann cell conditioned medium and the results of 2 microarrays.

We proposed 2 specific aims: 1.**Test the hypothesis that the loss of snf5 increases pain sensitivity by increasing the expression of the capsaicin receptor (TRPV1) in polymodal nociceptors and by inducing capsaicin-sensitivity in sensory neurons of other modalities.**

We have utilized a tomoxifin-inducible plpCre / snf5-flox/flox mouse to produce an *in vivo* targeted deletion of SNF5. We are using the expression of the capsaicin receptor, TRPV1, to estimate the number of nociceptors in sensory ganglia of these animals.

In experiments where we acutely ablated snf5 in adult mice, we detect an increase in the number of TRPV1-expressing neurons in the trigeminal and dorsal root ganglia (**figure 1**).

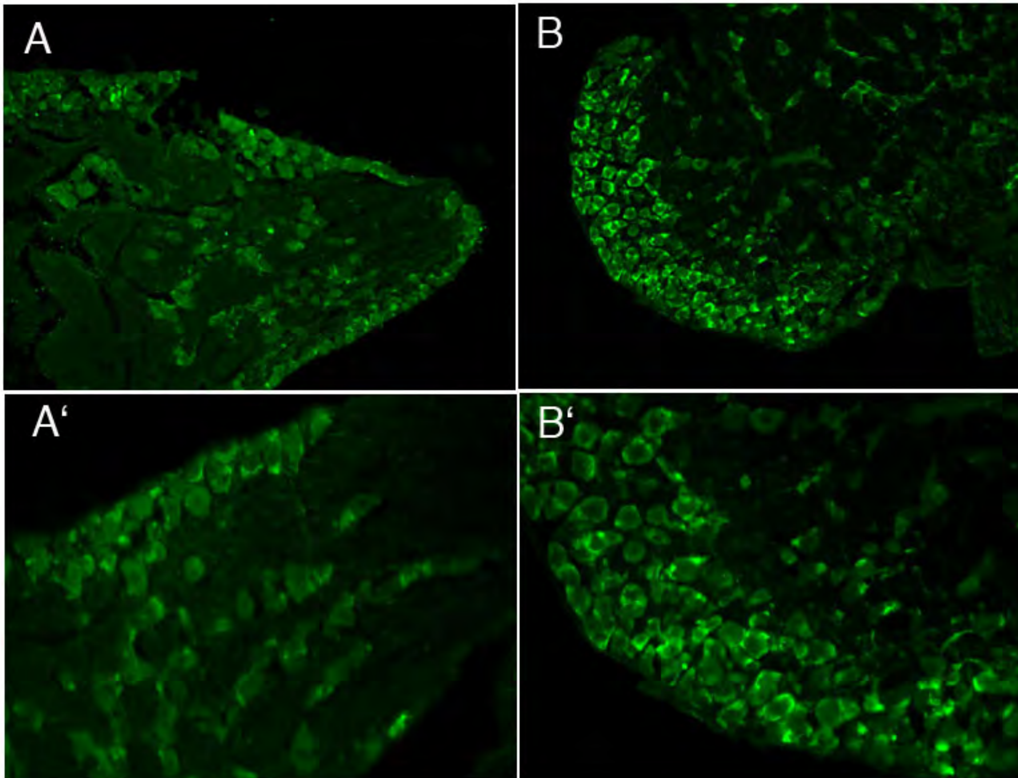

Figure 1. Trigeminal ganglion of oil injected (control; A,A') and tomoxifin injected (B, B') adult animals. Ganglia fixed 7 days after injections and lableled with anti-TRPV1. Panels are at 10X (A,B) and 20X (A', B').

To quantify the increase in TRPV1-IR neurons, we serially sectioned lumbar DRG (L1-5) and counted the neurons, ± TRPV1-IR, in every third section. The average percent positive neurons in 5 ganglia/animal were compared using a student's t-test. The results of 4 animals (2 tomoxifin-injected and 2 oil injected controls) were significantly different, 68 ± 9 vs 42 ± 6% respectively; $p< 0.01$

Strikingly, in preliminary cell counts, we observe an increase in large diameter (>24µm; 47% in tomoxifin- vs 8% oil injected controls) TRPV1-IR sensory neurons in lumbar DRGs (not shown) and trigeminal ganglia (**figure** 1). Since the small diameter (<20 µm) neurons are the typical nociceptors, this result suggests that sensory neurons of non-pain modalities are being recruited to express a nociceptor property. Consistent with this *in vivo* result, we detect an increase in large diameter, TRPV1-IR sensory neurons in cultures treated with conditioned medium (see figure 2 and Aim 2 below).

2. **Test the hypothesis that the loss of snf5 in Schwann and satellite cells results in an increase in their production and secretion of factors that increase the expression of TRPV1 in sensory neurons.**

In our second model we used an *in vitro* sensory neuron system to screen the biological activity of soluble factors released into the medium by snf5-knockout Schwann cells. Dorsal root ganglion (DRG) neurons of neonate to postnatal day 18 animals were dissociated and plated at a density of approximately 1000 neurons / cm^2. The base culture medium consists of L15, modified for 5% CO_2 atmosphere, as described in Mains and Patterson, (1973) with 5% adult rat serum, antibiotics and 100 ng/ml of 7s NGF.

The base medium was supplemented with medium conditioned by Schwann cells (SC-CM) ± snf5 (ablated using cre-lentivirual infection). The SC-CM was collected from confluent Schwann cell cultures, and concentrated using Amicon Ultra centrifugal filters with a 10000 molecular weight cutoff (Millipore). The SC-CM±snf5 was tested at 1-5 X concentration for 48-96 hours.

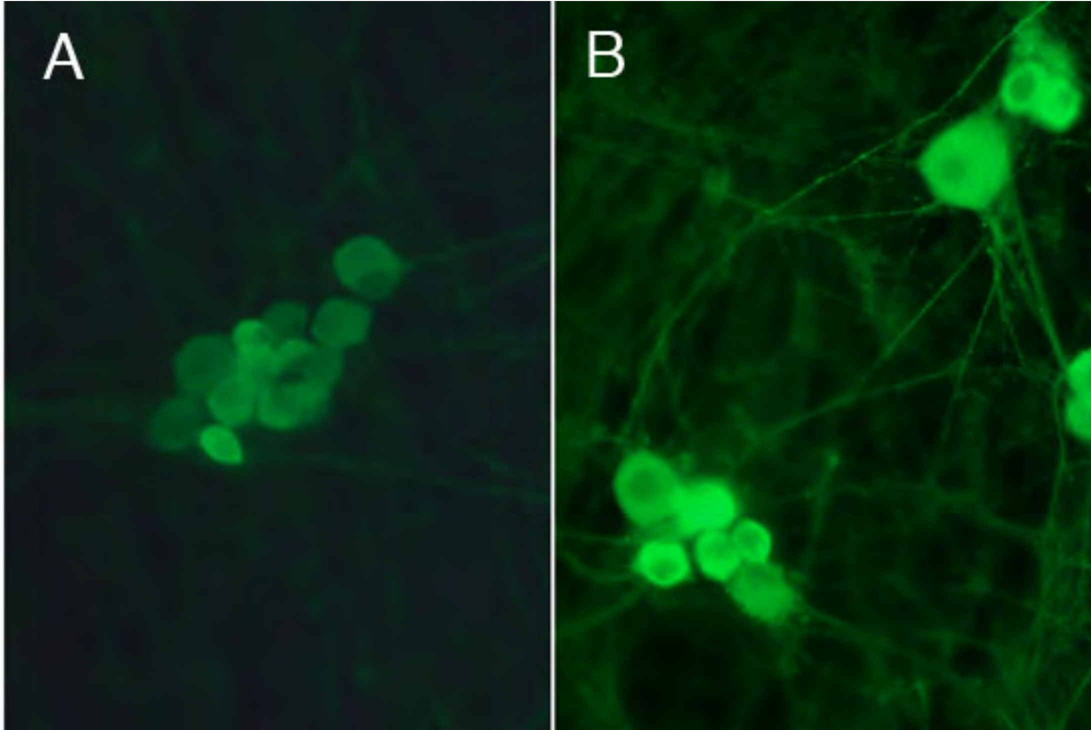

Figure 2. DRG culture treated with Control SC-CM (A) and -/- snf5 SC-CM (B) for 48 hours and labeled with anti-TRPV1. Note the presence of large diameter TRPV1-IR neurons in (B)

In these experiments, dissociated neurons were plated onto laminin-coated, 1 cm diameter glass coverslips. After CM treatment, the cultures were fixed and labeled with anti-TRPV1 antibodies and the appropriate secondary antibodies. For cell counts, each coverslip was divided into 4 sections and 3 randomly selected 20X fields were counted.

After 48 hours in CM we observe an increase in the percentage of TRPV1-IR neurons in cultures treated with -snf5 SC-CM (73 ± 7%) vs control SC-CM treatment (52 ± 2%). In addition, we observe both large and small diameter TRPV1-IR neurons in the snf5 -/-, SC-CM treatment but predominately small diameter neurons in the control SC-control CM, as we observed in the sensory ganglia *in situ* (not quantified at this time, figure 1)

We used a histochemical assay to determine the capsaicin sensitivity of sensory neurons cultured with ± *SNF5* SC-CM. In this assay, DRG cultures were incubated in a saline solution containing 0 calcium and 5 mM cobalt ± capsaicin (50 µM). After washing, intracellular cobalt was precipitated with ammonium sulfide. Following fixation, the cobalt precipitate was enhanced with silver using the Timm's intensification protocol (Matsumoto, 1994). Using this method, we detect a significant increase ($p < .01$) in capsaicin-sensitive neurons in –snf5 SC-CM (94 ± 1%) vs 58 ± 2% in control SC-CM treated cultures (figure 3)

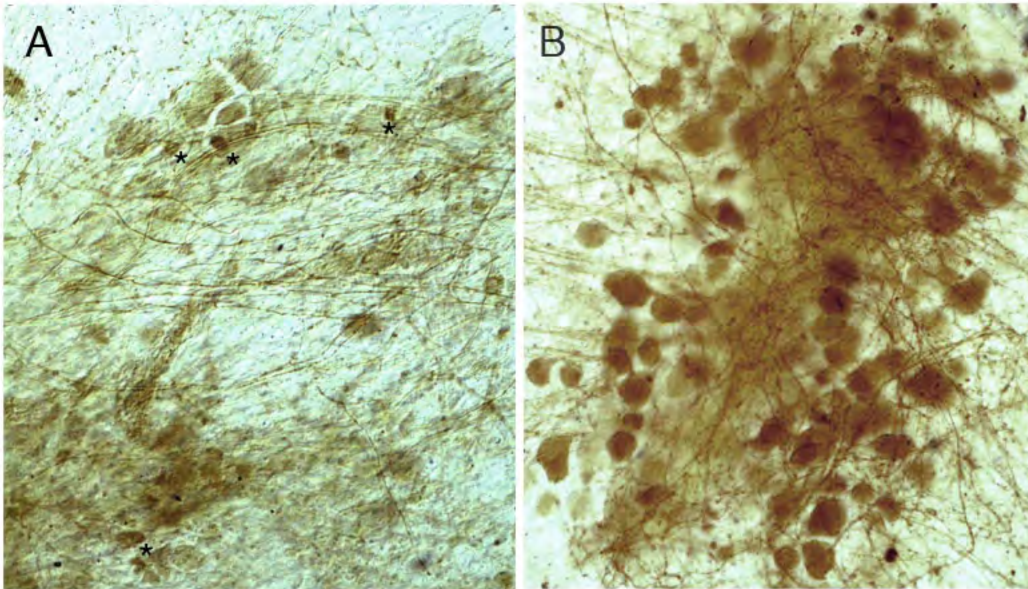

Figure 3. DRG culture, control SC-CM (A) and -snf5 SC-CM (B) treated with 50 μM capsaicin in the presence of 5 mM cobalt and subsequently enhanced with Timm's silver intensification protocol. Note, small diameter neurons labeled in (* in A) while many large and small diameter neurons labeled in (B).

Capsaicin Sensitivity can be Rapidly Enhanced by SC-CM. In a second series of culture experiments, we determined that the sensitivity of dorsal root ganglion neurons (as measured by cobalt uptake) increased after a 1 hour exposure to SNF5 -/- conditioned CM. Control cultures displayed 56% positive neurons vs 82% in CM-treated cultures ($p < 0.001$). This increase in capsaicin-sensitive neurons occurred in the absence of increased TRPV1-immunoreactivity.

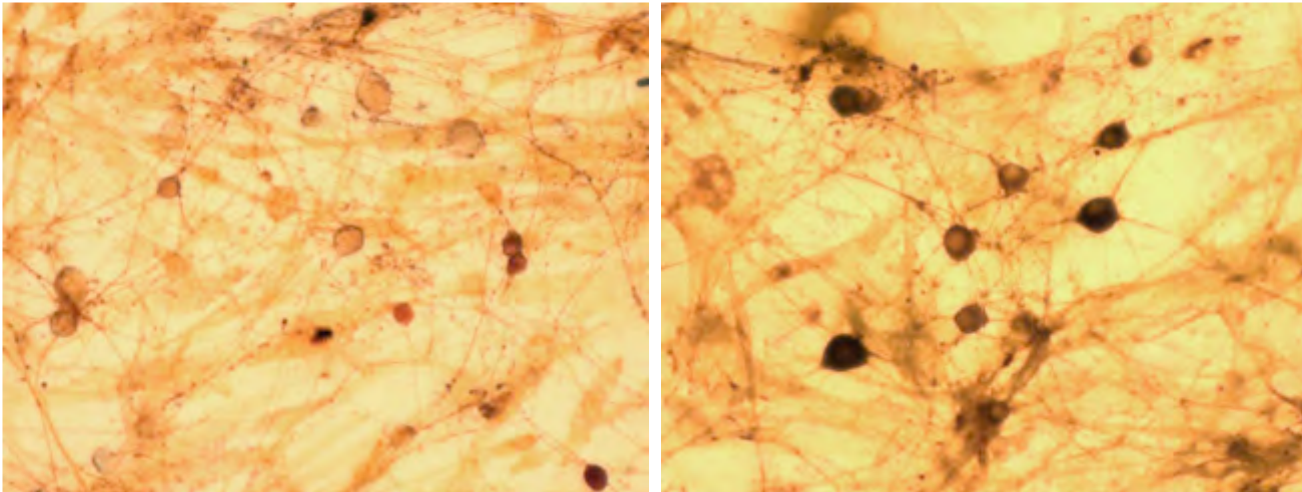

Figure 4. DRG culture treated with control SC-CM (left panel) and snf5SC-CM (right panel) for 1 hour. Both cultures were exposed to capsaicin 50 μM capsaicin in the presence of 5 mM cobalt and subsequently enhanced with Timm's silver intensification protocol as previously described.

Microarray analysis of Schwann cells ± snf5 and DRG sensory neurons treated with control and snf5-/- Schwann cell conditioned medium. Schwann cells from snf5 double-floxed animals were prepared in the following manner:

Sciatic nerves and brachial plexuses from P2-P5 snf5-/- double-floxed pups were dissected and dissociated with collagenase and trypsin treatment. Schwann cells are cultured in DMEM supplemented with 10% FBS, 100U/ml penicillin/streptomycin, 2ng/ml human herugulin-β1, 0.5 μM forskolin and 10ng/ml human bFGF.

To ablate snf5, the cultured cells were infected with either control or Cre- lentivirus. For the infection; 2x 10^5 Schwann cells were plated on D-polylysine coated 10 cm culture dish one day before infection. About 20 x10^5 transforming units (TU) were used for the infection (MOI 1:5-1:10). Culture medium was changed the next morning and the cells were maintained for another 2 to 3 days before collecting RNA.

The analysis of the microarrays data shows that the SNF5 does not affect the cell cycle. This result is in concordance with our in vitro data. The analysis does show an up regulation of several matrix matallopeptidases that are known to affect the development of tumors and tumor progression. Among these MMP 12, MMP 1a and MMP 10.
MMP 12 up 2.05
MMP 1a up 2.02
MMP 10 up 1.96
MMP 9 up 1.56
Our data also show an up-regulation of certain Chemokines /Chemokines receptors such as CCL5 (up 1.54) and CCr1(up 1.51).
We are continuing to analyze this data and identify possible links to our pain phenotype of enhanced capsaicin sensitivity,

Microarray of DRG sensory neurons. Wildtype DRG's were harvested from P7 pups and dissociated with collagenase/dispase. The neurons were preplated on uncoated culture plastic for 2 hours to reduce non-neuronal cells. The cells were gently resuspended and plated on to polylysine/laminin treated dishes. The culture medium contained 10 µM fUdr and uracil for 3 days. The neuron enriched cultures were treated with control or snf5-/- SC-CM for 24 hours and RNA collected.

The result of the neuron nicroarray is as follows:

Select transcripts up-regulated in DRG sensory neurons treated with CM from SNF5 -/_:

Transcripts	Fold change
RTP4 a Golgi chaperone (protects mu-delta receptors from ubiquitination and degradation).	1.7
ISG15 a tumor suppressor	1.84
Z-DNA binding protein1 role in the transport and translation of mRNA in axonal regeneration	1.55
Calcium channel voltage dependant alpha-2/delta subunit (Cacna2d1): Up regulated in neuropathic pain. Targeted by drugs used for focal epilepsy, neuropathic pain and anxiety desorders	1.35
Mu opioid receptors variant A	1.3
Sodium channel 207=Nav1.2	1.2
Glutamate receptor ionotropic AMPA2 (alpha2)	1.23

Among transcripts down-regulated in DRG sensory neurons treated with CM from SNF5 -/_:
4

Transcripts	Fold change
Ras homolog gene family, member A (Rhoa)	1.61
Poly(A) binding protein, cytoplasmic 4-like (Pabpc4l),	1.54
Versican (Vcan) Variant 2 (inhibit cell proliferation and tumor growth)	1.7
DNA cytosine methy transferase	1.99

As in the case of the Schwann cell array, we are continuing to analyze this data and identify possible links to our pain phenotype of enhanced capsaicin sensitivity,

Key Research Accomplishments.

Target-deletion of SNF5 leads to increase TRPV1 expression in sensory neurons
SNF5 -/- Schwann cells secrete a factor that stimulates the expression of TRPV1
The conditioned medium factor increases the capsaicin sensitivity of multiple classes of sensory neuron.
Conditioned medium from snf5-/- Schwann cells has long term effects (i.e. increased TRPV1 expression) and short term effects (i.e. rapid increase in capsaicin sensitivity). In the short term experiments, we did not detect a significant increase in the number of TRPV1-IR neurons in cultures that demonstrated increased capsaicin-sensitivity. This may be due to a lack of sensitivity in our immunocytochemistry or our inability to detect a redistribution of TRPV1 receptors (e.g. Zhang et al, 2005)

Reportable Outcomes.

Manuscript in preparation

Conclusion.

The preliminary identification of a conditioned medium factor (CM) that is >10K mw is interesting and potentially very important towards advancing our understanding of this disease. Our finding that the CM factor induces TRPV1 expression in large diameter sensory neurons is significant, since it suggests that one outcome of the mutation may be to convert non-pain sensory neurons to this phenoytype.

Our microarray analysis of Schwann cells ± snf5 and DRG sensory neurons treated with Schwann cell condition medium has identified potential secreted Schwann cell-derived factors and neuronal target genes.

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