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14. ABSTRACT

Neurofibromatosis Type I is cancer predisposition syndrome characterized by neurofibromas, benign tumors derived from peripheral nerve Schwann cells. The gene that is mutated to cause Neurofibromatosis Type I, NF1, encodes a 319 kDa protein called neurofibromin (NF1). NF1 negatively regulates members of Ras superfamily, oncogenes that are critical to the development of cancer. To gain insight into new NF1 functions, we performed experiments to identify the proteins with which it interacts. We found that NF1 associates with ribosomes suggesting the hypothesis that NF1 may regulate gene expression at the level of translation. We proposed to measure the levels of both mRNA and proteins for all genes in presence and absence of NF1 using the powerful and sensitive methods RNAseq, TRAPseq and SILAC mass spec analysis. These experiments are ongoing. We have completed the RNAseq of NF1 null iHSC-1λ ΔNF1#1 and scrambled control iHSC-1λ ScrP cells. This part of the project identified a substantial number of differentially expressed genes, suggesting that loss of NF1 is necessary and sufficient to suppress these genes at the level of transcription. Work of the total proteome and the TAPseq experiments are proceeding after preliminary experiments demonstrated the necessity that we switch to a DOX inducible NF1 expression system. This delay should not prevent us from testing the hypothesis that NF1 regulates expression at the level of translation. The results from these experiments will be a critical tool in understanding the function of NF1 and the consequences of NF1 mutation in the development of Neurofibromatosis Type I.

15. SUBJECT TERMS None listed.

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Introduction

We have made significant progress in achieving the goals of this proposal. As detailed below, we have made some small changes in our plans in light of recent experimental results. These changes should not affect our ability to test the hypothesis that NF1 regulates gene expression at the level translation.

Keywords

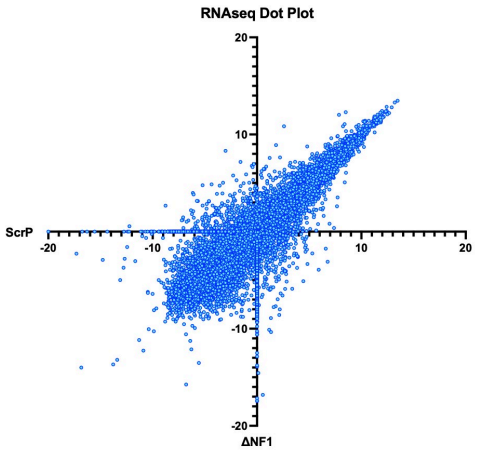
Neurofibromatosis Type 1, NF1, Neurofibromin, Transcription, Translation, Inflammation, Inflammosome.

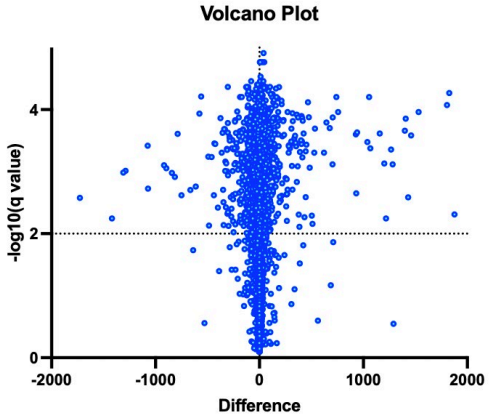
Accomplishments

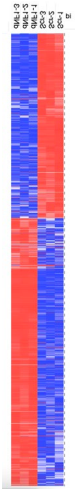
We have completed the RNAseq experiments described in Aim 1A. The total proteome experiments described in this aim are ongoing and we expect to have them complete by the end of June. One of the reasons for performing this experiment is that even in the event that our hypothesis proves false, we felt that an RNAseq experiment comparing control and NF1 knockout iHSC-1λ cells would generate interesting data. RNAseq identified more that 23,000 distinct transcripts expressed in either control iHSC-1λ^{Scr} or *NF1*-null iHSC-1λ$^{NF\#1}$ (Fig. 2A) . Of these, there are 1988 genes that are differentially expressed greater than two-fold relative to each other (p<=0.01, FDR = 1%) with 1173 induced in NF1 null cells relative to the scrambled gRNA control and 815 repressed relative to control. The RNAseq data is an essential reference for the proteome experiments that are designed to identify translationally regulated genes. However, the substantial number of differentially expressed genes suggests that loss of NF1 has significant effect on gene expression at the level of transcription as well.

Figure 1. RNAseq of NF1 null iHSC-1λ ΔNF1#1 and scrambled control iHSC-1λ ScrP cells

A. B. C.







D.

Gene	Scr-1	Scr-2	Scr-3	dNF1-1	dNF1-2	dNF1-3	P value	ΔNF1:Scr	Scr Rank%	ΔNF1 Rank %
IL6	2.65	2.23	2.64	25.86	26.22	27.25	<0.000001	10.55	51.9%	76.1%
COL4A1	36.39	39.07	42.12	254.01	258.20	247.45	<0.000001	6.46	81.3%	97.3%
POSTN	0.13	0.08	0.16	305.86	328.82	316.76	0.0000	2569.69	21.5%	98.1%
FN1	366.71	369.00	347.89	5049.40	4933.39	5055.84	<0.000001	13.88	98.3%	100.0%
CXCL12	171.62	123.89	119.16	785.52	780.07	782.84	0.0000	5.66	94.5%	99.2%

A. A dot plot display of expressed transcripts identified by triplicate RNAseq between NF1 null iHSC-1λ ΔNF1#1 and scrambled control iHSC-1λ ScrP cells. B. A volcano plot identifying significant differentially expressed transcripts (1% FDR). C. Hierarchical clustering of iHSC-1λ differentially expressed greater than 2-fold. D. A table showing transcript levels for IL6, COL4A1, POSTN, FN1 and CXCL12. Including p vole, Fold induction and expression level percentiles in each cell line.

We are still in the process of analyzing these data, but there are two types of differentially expressed genes that pique our interest, extracellular matrix (ECM) genes such as Collagen IV (COL4A1), Fibronectin (FN), and Periostin (POSTN) and cytokines like IL-6 and CXCL12 (Fig. 2D). This pattern of gene expression is interesting in light of recent publications showing that neurofibromas are characterized by large populations of immune cells[1-5]. The increased expression of ECM is among the earliest observations on the pathobiology of neurofibromatosis [6-9]. Our observations are consistent with this literature and suggest that loss of NF1 is necessary and sufficient to suppress these genes at the level of transcription. Since neurofibromas are characterized by both the presence of immune cells and increased ECM deposition, these data suggest that NF1 loss may induce a pattern of cytokine and ECM expression that induces an inflammatory response that is critical to neurofibromatosis pathology. Overexpression of cytokines may directly stimulate the immune system and various ECM proteins and their fragments are known to be pro-inflammatory as part of a normal would healing process, in chronic inflammatory disease and in a variety of cancers[10-12]. In fact, Schwann cell–

derived periostin has been shown to promote autoimmune peripheral polyneuropathy[13] and periostin mRNA expression is induced by ~2500 fold in NF1 knockout iHSC-1λ cells (Fig. 2D) and is overexpressed in NF1 null peripheral nerves[14]. These data may identify a novel mechanism for the initiation of inflammation in neurofibromatosis at the level of transcription.

Impact

Our observations are consistent with neurofibroma pathobiology and suggest that loss of NF1 is necessary and sufficient to suppress these pro inflammatory and ECM genes at the level of transcription. This highlights a more direct role for NF1 in regulating transcription, possibly via transcription factors activated by Ras in response to NF1 loss [15, 16]. This is also interesting in light of a publication that identifies NF1 as a transcriptional co-repressor for ERα [17] suggesting the possibility of a more direct role for NF1 in gene regulation. It will be interesting to identify transcripts that are restored by re-expression of NF in the NF1 null cell. It is too early to be definitive but these data do point to a path forward in the event that the hypothesis that NF1 regulates translation proved to be false.

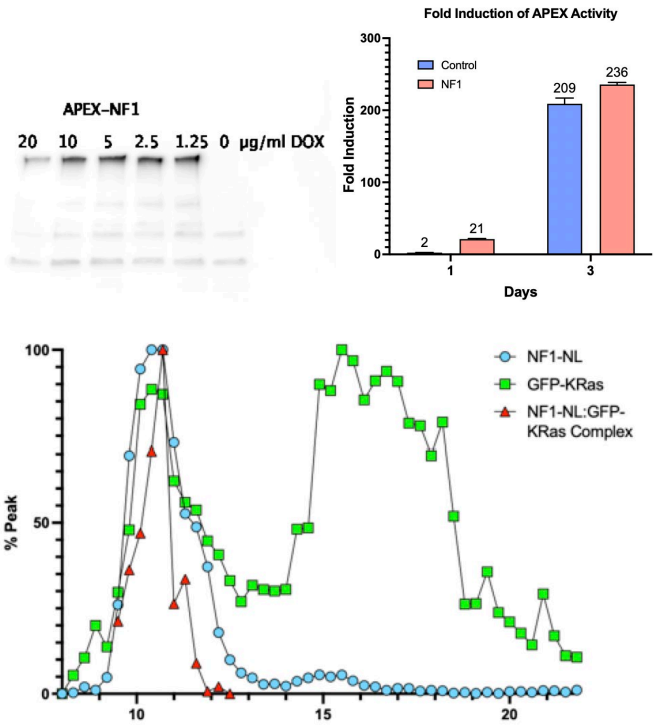
Changes/Problems

Our proposal leverages our ability to express full-length NF1 in immortalized human Schwann cells from a lentiviral expression system. This is challenging due to the size of the NF1 protein and its 8 kb cDNA. Given the commonly held belief that lentiviral vectors have an "upper size packaging limit" of 10 kb from 5'LTR to 3'-LTR, one would assume that NF1 cDNA would not fit into these vectors. However, publications in the literature showed that there is no hard cap at 10 kb but the packaging efficiency, and hence the titer, reduces by roughly five-fold for every kb above 10 kb and successful packaging of viral genomes of up to 18 kb have been achieved [18]. Since we are using lentiviral vectors with antibiotic selection markers, even the low titers we generate with lentiviral vectors expressing NF1 are sufficient to select for stable polyclonal populations of iHSC-1λ immortalized Schwann cells. We used this system to generate the preliminary mass spec and NF1 binding data we presented our proposal. However, in the intervening time, we found that NF1 expression gradually reduced with continued passage of these cell lines to the point it was too low to be useful. Ectopic silencing of lentiviral expression systems is not unknown but we do not see this effect in any of the other recombinant proteins we use with this system. This suggests there is a selective pressure against constitutive overexpression of NF1 in these cells, although we have not formally proved this.

To mitigate against this selective pressure, we generated cell lines with NF1 under control of a Doxycycline inducible lentiviral vector, pLVXT. We initially constructed a DOX inducible full-length NF1 fused to the proximity biotinylation enzyme APEX2. We are able to establish Dox inducible full length NF1 (Fig. 2A). We had to dramatically reduce the Dox concentration for the control cells to ensure an equivalent level of expression of the control and NF1 transgenes and in the course of this found that a 3-day incubation with DOX gave us maximal NF1 expression (Fig. 2B). To confirm that Dox induced NF1 is functional, we co-expressed GFP-KRas in

Figure 2 Dox Inducible NF1 Lentiviral System

A. B.



A. Immunoblot assay for NF1 expression with a dose response of Doxycycline.

B. Time course of NF1-APEX activity with Dox induction for 1 day and 3 days.

C. Size exclusion chromatography of lysates from Dox inducible APEX-NF1 cells co-expressing GFP-KRas. NF1-derived APEX activity (blue) co-elutes with GFP-Kras fluorescence (green) in the high molecular weight fractions. Immunoprecipitation of GFP-KRas from these reaction co-precipitates APEX-NF1 activity, (red) indicating the presence of NF1-KRas complexes.

the Dox inducible cell line and performed size exclusion chromatography to confirm full-length NF1 expression, we found that GFP-Kras co-migrated with APEX-NF1 n the high molecular weight fractions and that APEX-NF1 activity co-immunoprecipitated with GFP-KRas from these fractions (Fig. 2C) indicating that our ectopically expressed APEX-NF1 fusion protein binds to GFP-Kas and is therefore functional. These experiments validated our new NF1 expression system. We are in the process of making the DOX inducible GFP-NF1, GFP-NF1^{R1276G} and control GFP expressing iHSC1-λ NF1 knockout cells described for Aim 1B and Aim 2B. This is slightly challenging because both the Dox inducible system and the Cripsr system used for the knockout have a puromycin selectable marker, necessitating a change of one of these to a different selectable marker. Since the Tet3G expressing lentiviral vector has hygromycin resistance and bleomycin (Zeocin) has poor cytotoxic response in these cells we will replace the puromycin in the Dox inducible vector pLVXT with the BlastR gene to allow selection in blasticidin. These manipulations are time consuming but not difficult. Since we do not see lentiviral silencing in GFP-Rpl10 expressing iHSC-1l cell, the Trap-Seq experiments using these cells described in Aim 2A will be performed as described later this summer.

Products
None

Participants & Other Collaborating Organizations
None

Special Reporting Requirements
None

Appendices
None

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