# Therapeutic discovery for Friedreich ataxia using random shRNA selection

Avinash Kaur[1] , M. Grazia Cotticelli[1], Shujuan Xia[1], Fabio Acquaviva [2], Elisabetta Manduchi[1], Christopher Stoeckert[1], Yongping Wang[3], Robert B. Wilson[1]

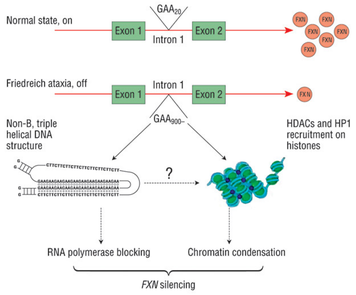
[1] *Perelman School of Medicine at the University of Pennsylvania, Philadelphia, United States*
[2] *Centro Chemis, Napoli, Italy*
[3] *Children's Hospital of Philadelphia, Philadelphia, United States*

## Abstract

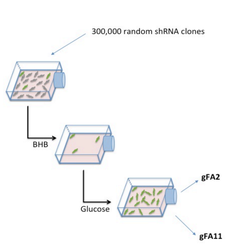
We screened a 300,000-clone, random shRNA-expressing library and identified shRNA sequences that reverse the decreased growth/survival phenotype of primary Friedreich ataxia (FA) fibroblasts grown in mitochondrial stress media. One of the hit sequences, gFA2, increases frataxin expression ~2 fold, either as a vector-expressed shRNA or as a transfected siRNA. We randomly mutagenized gFA2 to create a gFA2 variant sub-library. We screened this sub-library in primary FA fibroblasts and identified two gFA2 variants, gFA2.8 and gFA2.10, that further increase frataxin expression. Microarray analyses of primary FA fibroblasts expressing another hit shRNA, gFA11, revealed alterations in ~350 mRNAs. Bioinformatic pathway analyses indicated significant changes in mRNAs involved in cytokine secretion; we confirmed significant changes in cytokine secretion induced by gFA11 biochemically. Ingenuity Pathway Analysis revealed that inhibition of a known transcription factor, or treatment of cells with a previously studied chemical compound, induced a statistically similar pattern of gene expression to that induced by gFA11. Inhibition of the transcription factor using a directed siRNA in primary FA fibroblasts, as well as treatment of the cells with the chemical compound, recapitulated the phenotype induced by gFA11, namely reversal of decreased growth/survival in mitochondrial stress media. We are currently planning similar microarray and bioinformatics analyses of the optimized versions of gFA2. Combined with microarray analyses and bioinformatic pattern-matching, our random, shRNA library screens potentially yield, **1) small-RNA therapeutic candidates**, **2) conventional chemical-compound therapeutic candidates**, **3) drug-target candidates**, and **4) elucidation of disease mechanisms**, which may inform additional therapeutic initiatives.

## Background

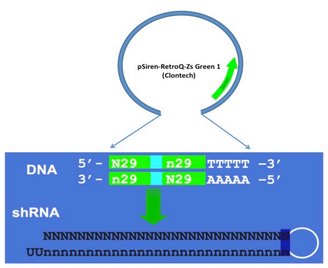
Friedreich ataxia (FRDA) is an autosomal recessive neuro and cardio-degenerative disorder, with a prevalence of approximately 1 in 40,000 in European populations. (Recent reviews include those by Koeppen and Mazurkiewicz,1 Collins,2 and Gomes and Santos.3 ) FRDA is characterized by progressive ataxia of all four limbs, dysarthria, areflexia, sensory loss, and muscle fatigability. FRDA is caused by mutations in the nuclear gene, FXN , which encodes the highly conserved protein frataxin. Most disease alleles harbor a GAA repeat expansion in the first intron, which results in decreased transcription. Frataxin localizes primarily to the mitochondrial matrix, where it chaperones iron and regulates the iron–sulfur cluster (ISC) assembly complex.




## Library Screening



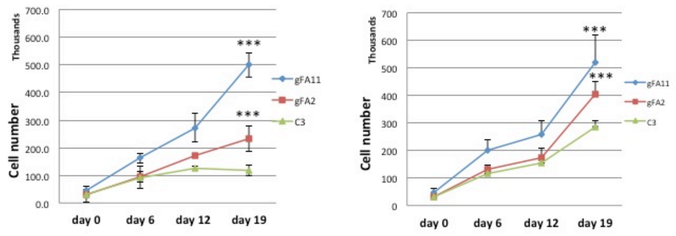

**Library screening:**
We infected primary FA fibroblasts (Coriell GM3816) with a 300,000-clone, random shRNA library. Four days post-infection the cells were switched to DMEM containing 5 mM BHB. After two weeks, only 1% of the cells survived. The cells were then moved into DMEM with glucose for two weeks. The entire cycle was repeated two more times. Finally, we extracted genomic DNA from the selected cells, amplified the shRNA-expressing cassette by PCR, and cloned the amplicon back into the parental vector. Two clones were highly represented and chosen for further analysis: **gFA2** and **gFA11**.




## Results

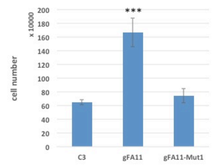
**Clones gFA2 and gFA11 increase the growth of FA fibroblasts**

FA 3665B cells were infected with gFA2 (squares), gFA11 (diamonds) or a random clone (triangles). Cells were then sorted and GFP-positive cells were grown in DMEM plus BHB 5 mM (left) or in DMEM plus 5 mM glucose (right) ***= p < 0.05




## Candidate 1: gFA11

**Design of a mutant of gFA11 associated with loss of the growth phenotype**



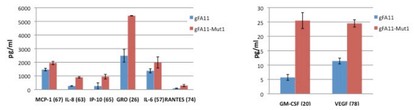

**gFA11-siRNA sequence**
XXXXGXXXXXXXXXXXXXXXXXdGdG
UUXXXXCXXXXXXXXXXXXXXXXX C C

**gFAMut1- siRNA sequence**
XXXXCXXXXXXXXXXXXXXXXXdGdG
UUXXXXGXXXXXXXXXXXXXXXXX C C

**We performed microarray analysis of GM3816 cells infected with gFA11 vs. cells infected with the Mut1 siRNA**

**D.A.V.I.D. analysis of 301 genes in the microarray data suggested that gFA11 changed the expression level of many genes associate with "secretion."**

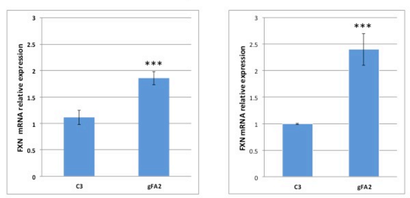

**We collected conditioned media from FA cells transfected with gFA11 or control (Mut 1) and confirmed by Luminex analysis that gFA11 affects the secretion of cytokines.**




## Candidate 2: gFA2

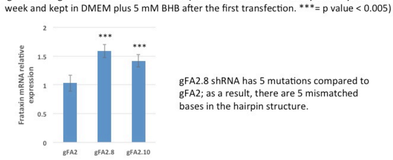
**gFA2 increases frataxin expression in FA fibroblasts as shRNA or siRNA**

Frataxin expression in GM3816 cells infected with gFA2 vs. a random clone (left) and in GM3665B cells transfected with 10 nM gFA2 siRNA or a random clone (right). Cells were transfected every 3-4 days for two weeks and kept in BHB medium after the first transfection. ***= p value < 0.005




**Hit-optimization of clone gFA2**

We constructed a sub-library of ~3000 sequences, each with a limited number of random mutations compared to gFA2. We screened this sub-library as described previously and identified two gFA2-variant clones, gFA2.8 and gFA2.10. Frataxin expression in primary FA fibroblasts (4675) transfected with 10 nM siRNA of each sequence demonstrates that clones gFA2.8 and gFA2.10 are more efficacious. (Cells were transfected every two days for one week and kept in DMEM plus 5 mM BHB after the first transfection. ***= p value < 0.005)




gFA2.8 shRNA has 5 mutations compared to gFA2; as a result, there are 5 mismatched bases in the hairpin structure.

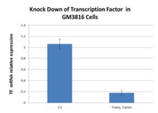
gFA2: XXXAAXXXXXG XXXXXXXXC XXXXXXX (CTAAAC) CXXXXXXXXXXXXXXXXXXXXXXX
gFA2.8: XXXCCXXXXC XXXXXXXA XXXXXXX (CTAAAC) TXXXXXXXXXXXXXXXXXXXXXXX

## Materials and Methods

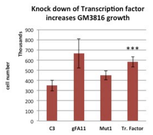
GM3816 and GM3665B FA cells were from Coriell; Patient derived cells 4657 were a gift of Dr. David Lynch (Children's Hospital of Philadelphia, PA, USA); Microarray analysis used the Affymetrix GeneChip Human Gene 2.0 ST Array.
Luminex analysis was performed by the Human Immunology Core Facility at University of Pennsylvania using the Milliplex Panel HCYTOMAG-60K kit (Millipore).
For the Bioinformatic analyses: IPA was run through the Penn Genomic Analysis Core; we used the free online available version of the **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery (**DAVID**) v6.7 and **G**ene **S**et **E**nrichment **A**nalysis from the Broad Institute (MA).

## Identification of Drug Target and Drug

**Ingenuity Pathway Analysis (IPA) of microarray data**

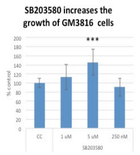



IPA identified a transcription factor as a negative upstream regulator (activation z-score of -4.26). We knocked down this transcription factor using a directed siRNA and recapitulated the growth phenotype seen with gFA11.
(GM3816 were transfected with 10 nM siRNA every 3-4 days for one week.)




**Ingenuity Pathway Analysis (IPA) of microarray data**

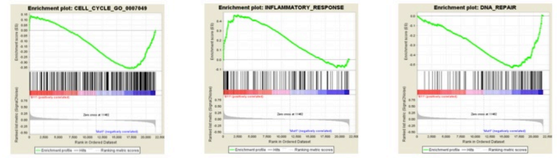
SB203580, a MAP38 kinase inhibitor, had an activation z-score of +3.14. We tested this drug at different concentrations and found that it was able to increase the growth of FA cells in a dose-dependent manner.
(Cells were treated with the drug or carrier control (DMSO) every 48 h for two weeks.)




## Elucidation of Disease Mechanisms

**G.S.E. analysis of microarray data**

**Enrichment in phenotype: gFA11 (3 samples)**
403 / 577 gene sets are upregulated
115 gene sets are significant at FDR < 25%
**60** gene sets are significantly enriched at nominal p-value < 1%




The pathways identified by GSE Analysis are currently under investigation.

## References

Wang et al. Plos One 2008 3(9)
Huang DW et al. Nature Protoc. 2009; 4(1):44-57
Huang DW et al. Nucleic Acids Res. 2009; 37(1):1-13.
Subramanian et al. PNAS 2005; 102: 15545-15550
Mootha et al. Nat Genet. 2003; 34:267-273
Pandolfo, Archives of Neurology, 2008
Cotticelli et al. JBMS 2015