Assays to Measure Downstream Consequences of Frataxin Deficiency

In Friedreich's ataxia (FRDA), large expansions of the intronic, triplet GAA repeat in the *FXN* gene result in reductions in the protein product Frataxin (FXN). FXN is a small, highly conserved mitochondrial protein, whose loss impairs iron-sulfur cluster (ISC) biosynthesis. In FRDA patients, disrupted ISC biosynthesis causes maladaptive alterations in cellular processes downstream of ISC biogenesis that manifest as neurodegeneration, hearing and vision loss, diabetes, and fatal cardiomyopathy. The extent of GAA expansion and consequent FXN loss strongly correlate with age of disease onset, disease severity, and age at death in FRDA patients.

Direct assessment of FXN levels, particularly in affected tissues that are difficult to access, has been challenging for the field, so researchers have developed assays to measure downstream consequences of reduced FXN expression and function. These include activity of various ISC containing proteins and assays for metabolic changes related to reduction in ISC protein activities, as well as oxidative damage and mitochondrial function deficits, as described below. Additional assays are also being developed that relate to other processes disrupted in FRDA cells.

This is a list of assays that have been used as downstream measures of FXN, but many other markers are also used.

ISC-Containing Proteins

A plethora of proteins require ISCs for functions such as enzymatic activity, protein stability and protein-protein interactions. The expression and activity of a handful of these ISC-containing proteins have been used as surrogate markers of FXN levels.

Metabolic Enzymes, such as subunits of the mitochondrial oxidative phosphorylation (OXPHOS) complexes (i.e., complex I NADH dehydrogenase, complex II succinate dehydrogenase, and complex III cytochrome c reductase) have reduced activity (and sometimes expression) in the context of Fe-S cluster deficit (i.e. FRDA conditions). Additionally, the Krebs cycle enzyme aconitase has reduced activity under Fe-S cluster deficiency conditions. As a last example, the presence of lipoic acid (detected by an antibody against LA) on specific enzymes (e.g., pyruvate dehydrogenase, PDH and 2-oxoglutarate dehydrogenase, OGDH) is reduced due to the reduced activity of ISC-containing lipoic acid synthase under Fe-S cluster deficit.

Several DNA repair enzymes also contain ISC clusters, and their activity is impacted in FRDA due to Fe-S cluster deficits. The levels of DNA damage seen in cells , the expansion of the repeat sequence itself and the activity of the repair enzymes have been used as measurements of impairment in FRDA cells.

Please consult the following example references for FRDA conditions, as well as assay protocols to measure expression and activity of ISC-containing proteins.

Metabolic Enzymes

- **FRDA Patient Tissue**
  - Fresh tissue biopsy. Rötig et al. (1997) "Aconitase and mitochondrial iron-sulfur protein deficiency in Friedreich ataxia." *Nature Genetics*. **17:** 215-7.
  - Frozen tissue biopsy. Bradley et al. (2000) "Clinical, biochemical and molecular genetic correlations in Friedreich's ataxia." *Human Molecular Genetics*. **9:** 275-82.
- **FRDA Animal Models**
  - Differentiated neurons derived mouse neural stem cells from the humanized YG8R mouse. Sandi et al. (2014) "Generation and characterization of Friedreich ataxia YG8R mouse fibroblast and neural stem cell models." *PLoS One*. **9:** e89488.
  - MCK-FXN knockout mouse model (i.e., heart and skeletal muscle-specific FXN deletion). Puccio et al. (2001) "Mouse models for Friedreich ataxia exhibit cardiomyopathy, sensory nerve defect and Fe-S enzyme deficiency followed by intramitochondrial iron deposits." *Nature Genetics*. **27:** 181-6.
  - FRDA knockdown mouse model (i.e., systemic FXN loss). Chandran et al. (2017) "Inducible and reversible phenotypes in a novel mouse model of Friedreich's Ataxia." *eLife*. **6:** e30054.
  - FRDA *Drosophila* model (re: systemic FXN reduction). Kondpalli et al. (2008) "*Drosophila* frataxin: An iron chaperone during cellular Fe-S cluster bioassembly." *Biochemistry*. **47:** 6917-27.
  - Reduction of Caenorhabditis elegans frataxin increases sensitivity to oxidative stress, reduces lifespan, and causes lethality in a mitochondrial complex II mutant. Vázquez-Manrique RP, González-Cabo P, Ros S, Aziz H, Baylis HA, Palau F. FASEB J. 2006 Jan;20(1):172-4. Epub 2005 Oct 24.
  - Understanding the genetic and molecular pathogenesis of Friedreich's ataxia through animal and cellular models. Martelli A, Napierala M, Puccio H. Dis Model Mech. 2012 Mar;5(2):165-76. D 10.1242/dmm.008706. Review.
- **FRDA cell culture models, fibroblasts, derived iPSCs and iPSC-differentiated cells**
  - Fibroblasts and iPSCs. Hick et al. (2013) "Neurons and cardiomyocytes derived from induced pluripotent stem cells as a model for mitochondrial defects in Friedreich's ataxia." *Disease Models and Mechanisms*. **6:** 608-21.
    - Found no evidence of ISC defects in these models.
  - iPSC-differentiated neurons. Codazzi et al. (2016) "Friedreich ataxia-induced pluripotent stem cell-derived neurons show a cellular phenotype that is corrected by a benzamide HDAC inhibitor." *Human Molecular Genetics*. **25:** 4847-55.
    - Found reduced ISC protein expression in this model.
  - Using human pluripotent stem cells to study Friedreich ataxia cardiomyopathy. Crombie DE, Pera MF, Delatycki MB, Pébay A. Int J Cardiol. 2016 Jun 1;212:37-43. doi: 10.1016/j.ijcard.2016.03.040. Epub 2016 Mar 21. Review.

DNA Repair Enzymes

- **FRDA cell culture models, fibroblasts, derived iPSCs and iPSC-differentiated cells**
    - Fibroblasts from YG8 mice. Khonsari et al. (2016). "Lentivirus-meditated frataxin gene delivery reverses genome instability in Friedreich ataxia patient and mouse model fibroblasts." *Gene Ther.* Dec;23(12):846-856.
    - Fibroblasts. Bhalla et al. (2016). "Deep sequencing of mitochondrial genomes reveals increased mutation load in Friedreich's ataxia." *Ann Clin Transl Neurol.* 14;3(7):523-36
- **FRDA animal models**
    - YG8 mice. Ezzatizadeh et al. (2014). "MutLα heterodimers modify the molecular phenotype of Friedreich ataxia." *PLoS One.* 27;9(6)
    - KIKO mice. She et al. (2016). "Frataxin Deficiency Promotes Excess Microglial DNA Damage and Inflammation that Is Rescued by PJ34." *PLoS One.* 8;11(3)

Bioenergetics

FXN is required for the function of ISC-containing proteins as described above. Several important ISC-containing proteins are involved in the electron transport chain or in the Krebs cycle. This means that one consequence of FXN deficiency is the alteration of bioenergetics and cellular energy metabolism. Many assays have been established to study energy metabolism in vivo and in vitro, and some of these have been used to measure downstream effects of FXN deficiency in FRDA patients and in animal models of the disease.

- **In FRDA patients:**
    - Lodi R, Cooper JM, Bradley JL, Manners D, Styles P, Taylor DJ, Schapira AHV. (1999) "Deficit of in vivo mitochondrial ATP production in patients with Friedreich ataxia." *PNAS.* 96:11492-5. Calf muscle.
    - Vorgerd M, Schöls L, Hard C, Ristow M, Epplen JT, Zange J. (2000) "Mitochondrial impairment of human muscle in Friedreich ataxia in vivo." *Neuromuscul. Disord.* 10:430-5. Calf muscle
    - Lynch DR, Lech G, Farmer JM, Balcer LJ, Bank W, Chance B, Wilson RB. (2002) "Near Infrared muscle spectroscopy in patients with Friedreich's ataxia." *Muscle Nerve.* 25:664-73. Calf muscle
    - Bossie HM, Willinham TB, Schoick RAV, O'Conner PJ, McCully KK. (2017) "Mitochondrial capacity, muscle endurance, and low energy in Friedreich ataxia." *Muscle Nerve.* 56:773-9. Forearm muscle
    - Worth AJ, Basu SS, Deutsch EC, Hwang WT, Snyder NW, Lynch DR, Blair IA. (2015). "Stable isotopes and LC-MS for monitoring metabolic disturbances in Friedreich's ataxia platelets." *Bioanalysis.* 7(15):1843-55. Patient derived platelets

- **In FRDA animal models**
    - Abeti R, Parkinson MH, Hargreaves IP, Angelova PR, Sandi C, Pook MA, Giunti P, Abramov AV. (2016) "Mitochondrial energy imbalance and lipid peroxidation cause cell death in Friedreich's ataxia." *Cell Death Dis.* 7: e2237. YG8R mouse

- Martin AS, Abraham DM, Hershberger KA, Bhatt DP, Mao L, Cui H, Liu J, Liu X, Muehlbauer MJ, Grimsrud PA, Locasale JW, Payne RM, Hirschey MD (2017) "Nicotinamide mononucleotide requires SIRT3 to improve cardiac function and bioenergetics in a Friedreich's ataxia cardiomyopathy model" *JCI Insight*. 2017 Jul 20;2(14). MCK mouse
- Review. Schipara, A., and Lodi, R. (2004) "Assessment of in vitro and in vivo mitochondrial function in Friedreich's ataxia and Huntington's disease." *Methods Mol Biol.* 277:293-307.

Oxidative Damage, Iron Dysregulation and Antioxidant Pathways

Impairment of ISC biogenesis and consequent inactivation of OXPHOS ISC proteins is thought to contribute to the generation of reactive oxygen species (ROS), which may cause cellular oxidative damage. Additionally, FXN is thought to regulate a cysteine desulfurase in the formation of Fe-S clusters, which in turn disrupts iron homeostasis. As such, loss of FXN results in alterations of cellular iron homeostasis, which may also contribute to oxidative stress. As a consequence, FXN loss results in compensatory shifts in cellular antioxidant defense pathways, which includes depletion of glutathione levels and enhanced expression of mitochondrial superoxide dismutase (SOD2). Furthermore, FXN deficiency causes reduced expression of transcription factor Nuclear factor erythroid 2-related factor 2 (NRF2), which limits the ability of cells to respond to oxidative stress. Both direct measures of ROS and oxidative damage and these antioxidant compensatory shifts have serves as indicators of FXN loss in certain models.

For a reviews of oxidative stress in FRDA and assay protocols to measure oxidative damage and antioxidant pathways, please consult the following references:

- **Oxidative damage assays in animal and cell models**
  - Lupoli et al. (2017) "The role of oxidative stress in Friedreich's ataxia" *FEBS Letters.* **592:** 718-27.
  - Cotticelli et al. (2013) "Insights into the role of oxidative stress in the pathology of Friedreich ataxia using peroxidation resistant polyunsaturated fatty acids." *Redox Biol.* **1**:398-404

Mitochondrial Morphology and Function

Downstream of FXN loss, several pathways regulating mitochondrial morphology and health are negatively impacted. Mitochondrial dysfunction has been hypothesized to give rise to FRDA tissue pathology and clinical presentation, including neurodegeneration and hypertrophic cardiomyopathy. Although the precise relationship between mitochondrial morphological alterations and dysfunction remain to be fully elucidated, FRDA mitochondrial dysfunction entails shifts in mitochondrial content (i.e., mitochondrial loss in neurons and mitochondrial accumulation in heart cells). Moreover, FXN deficiency causes altered expression of the mitochondrial biogenesis master regulator PGC1α, reduced mitochondrial membrane potential, and perturbed mitochondrial

proteostasis. These alterations can serve as indicators of reduced FXN expression and activity, as described in the sample literature below:

- **FRDA Animal Models**
  - Humanized YG8R mouse (re: neuronal mitochondrial content and membrane potential). Abeti et al. (2016) "Mitochondrial energy imbalance and lipid peroxidation cause cell death in Friedreich's ataxia" *Cell Death & Disease.* **7:** e2237.
  - FXN knock-in/knockout mouse model (re: neuronal mitochondrial biogenesis). Lin et al. (2017) "Early cerebellar deficits in mitochondrial biogenesis and respiratory chain complexes in the KIKO mouse model of Friedreich ataxia." *Disease Model Mechanisms*. **10:** 1343-52.
  - MCK-FXN knockout mouse model (re: cardiac mitochondrial ultrastructure). Puccio et al. (2001) "Mouse models for Friedreich ataxia exhibit cardiomyopathy, sensory nerve defect and Fe-S enzyme deficiency followed by intramitochondrial iron deposits." *Nature Genetics*. **27:** 181-6.
  - MCK-FXN knockout mouse model (re: mitochondrial protease expression). Guillon et al. (2009) "Frataxin deficiency causes upregulation of mitochondrial Lon and ClpP proteases and severe loss of mitochondrial Fe-S proteins." *FEBS Journal.* **276:** 1036-47.
  - FRDA knockdown mouse model (re: cardiac and neuronal mitochondrial ultrastructure). Chandran et al. (2017) "Inducible and reversible phenotypes in a novel mouse model of Friedreich's Ataxia." *eLife*. **6:** e30054.
  - FRDA *Drosophila* model (re: axonal membrane potential). Shidara et al. (2010) "Defects in mitochondrial axonal transport and membrane potential without increased reactive oxygen species production in a Drosophila model of Friedreich ataxia," *The Journal of Neuroscience*. **30**: 11369-78.
  - Review. Schipara, A., and Lodi, R. (2004) "Assessment of in vitro and in vivo mitochondrial function in Friedreich's ataxia and Huntington's disease." *Methods Mol Biol.* 277:293-307.

- **FRDA cell culture models**
  - iPSC -cardiomyocytes and -neurons (re: mitochondrial ultrastructure and membrane potential, respectively). Hick et al. (2013) "Neurons and cardiomyocytes derived from induced pluripotent stem cells as a model for mitochondrial defects in Friedreich's ataxia." *Disease Models and Mechanisms*. **6:** 608-21.
  - iPSC-cardiomyocytes (re: mitochondrial content and ultrastructure). Lee et al. (2013) "Modeling of Friedreich ataxia-related iron overloading cardiomyopathy using patient-specific-induced pluripotent stem cells." *Pflügers Archiv: European Journal of Physiology.* **466:** 1831-44.
  - iPSC-differentiated neurons (re: mitochondrial protease expression). Codazzi et al. (2016) "Friedreich ataxia-induced pluripotent stem cell-derived neurons show a cellular phenotype that is corrected by a benzamide HDAC inhibitor." *Human Molecular Genetics*. **25:** 4847-55.

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