Testing five hypotheses for Friedreich's ataxia in human cells

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Overview:

Friedreich ataxia– a ‘mitochondrial oxidative stress’ disease

Friedreich’s ataxia pathology

Testing 5 hypotheses by microarray

Biochemical confirmations of one model

Causes and effects of oxidative stress

Avenues to therapy

Request for cells

Features of Friedreich's Ataxia

- 1/50,000 incidence
- Commonest inherited ataxia
- Autosomal recessive inheritance
- Triplet repeat disease
- Spinal neuron degeneration
- Phenotype similar to Vitamin E transporter deficiency

Spinal cord dorsal root ganglion cells degenerate

Control

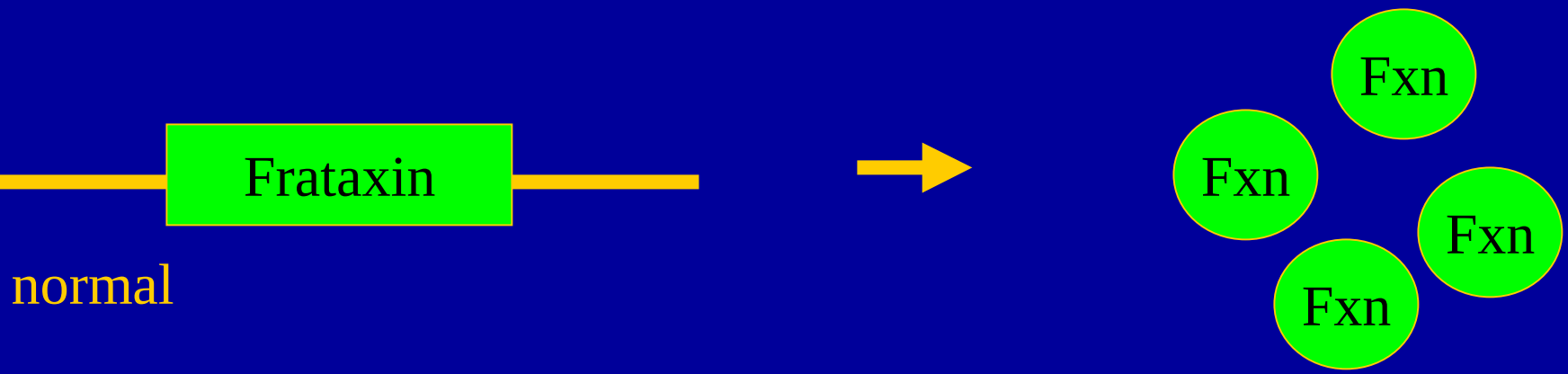
Patient

Friedreich's ataxia pathology--heart

- Hypertrophic cardiomyopathy
- Cardiomyocyte degeneration
- Cardiac accumulation of iron and lipofuscin

What causes Friedriech's ataxia?

Expansions of GAA reduce frataxin expression

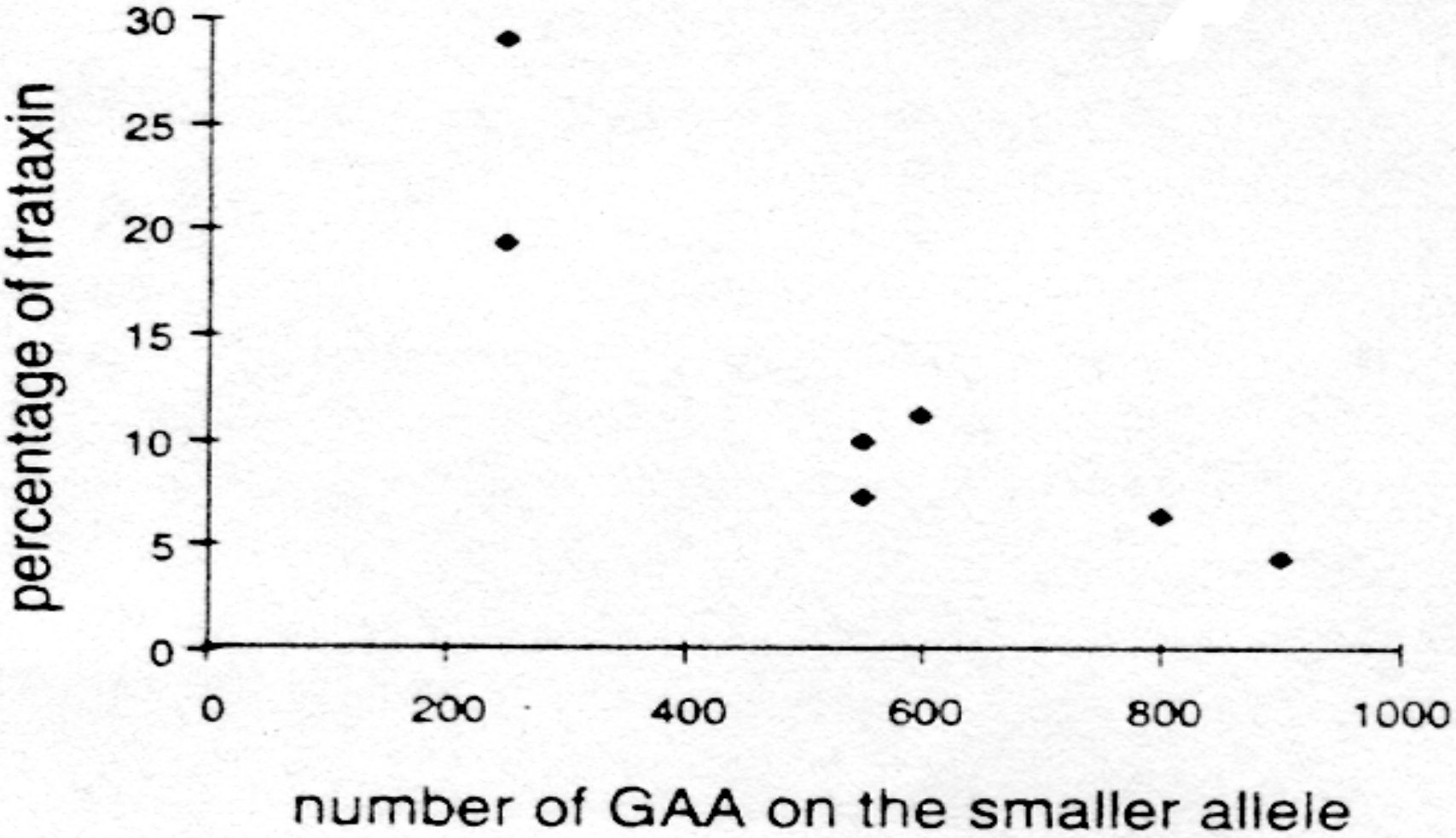


normal

GAAGAAGAAGAAGAAGAAn

mutant

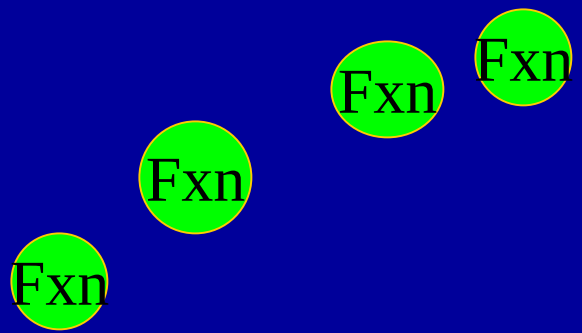
Larger expansions, less frataxin, more severe disease



Campuzano et al. 1997, HMG

What is frataxin?

Frataxin is a nuclear-encoded gene expressed in mitochondria



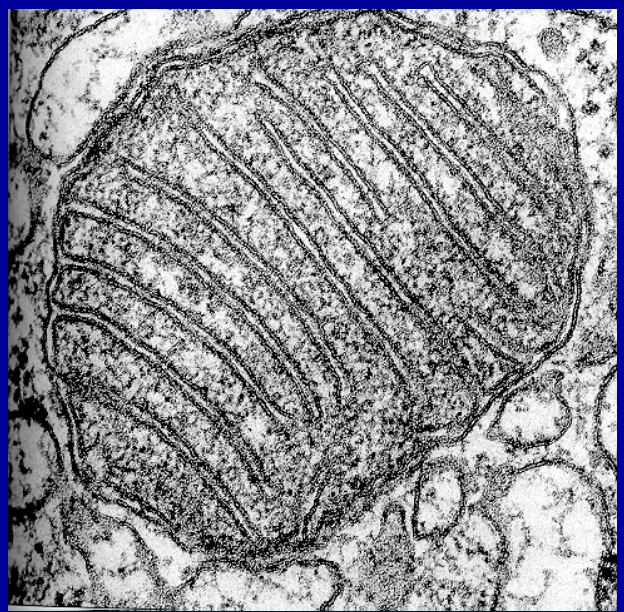
What are Mitochondria?
Mitochondria are plentiful in most human cells

Mitochondria participate in many pathways

Energy Generation

Apoptosis

Ca++ Buffering



Nucleotide synthesis/salvage

Urea cycle

Fatty acid oxidation

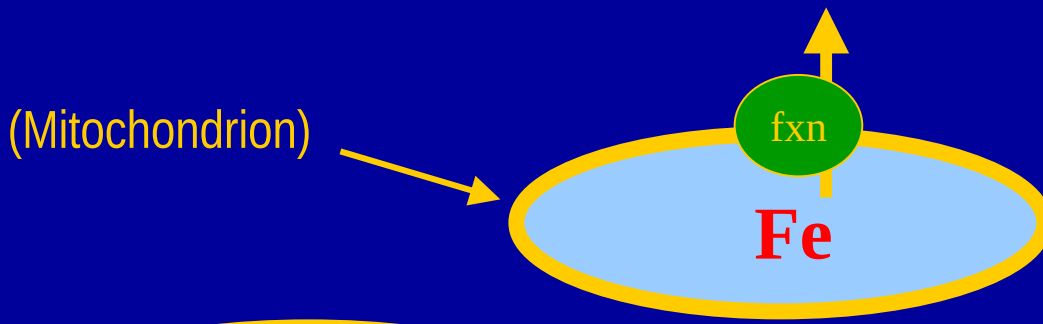
Heme Synthesis

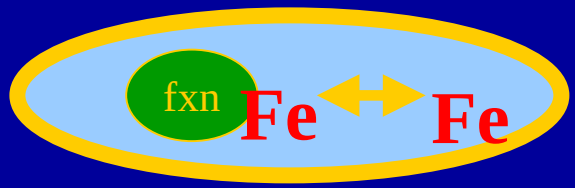
Two points have dominated frataxin research

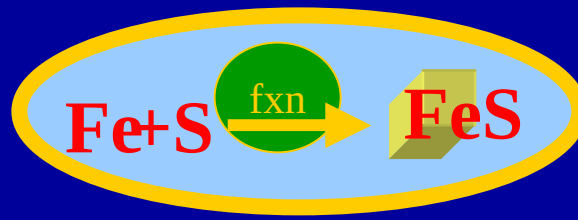
- Question 1: What is frataxin's correct function in human cells?
- Question 2: How does frataxin loss cause disease?

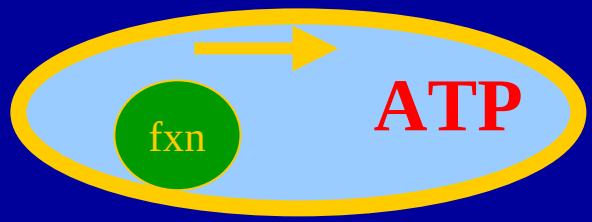
There are 5 potential functions of frataxin based on work in models:

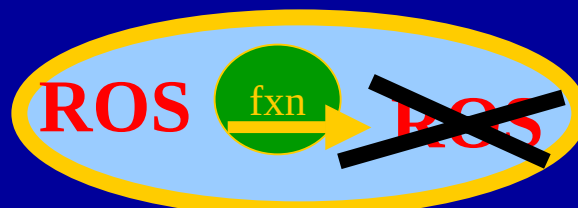
- iron transporter
- iron-binding protein
- Fe-S cluster assembler
- Oxphos stimulator
- Mitochondrial antioxidant











We study the effects of frataxin-deficiency in human cells

- 5 Hypotheses for frataxin's real function have been generated from work in Yeast, Bacteria, and Mice
- However Friedriech's ataxia is a human disease
- Therefore we wanted to test which function for frataxin is most likely in human cells

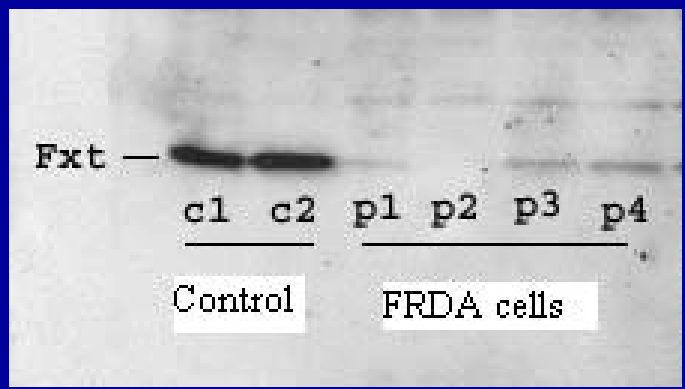
Human cell models of Friedreich's ataxia:

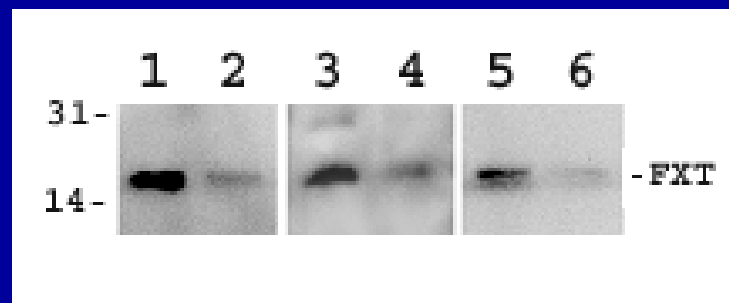
Patient Fibroblasts

Patient Lymphoblasts

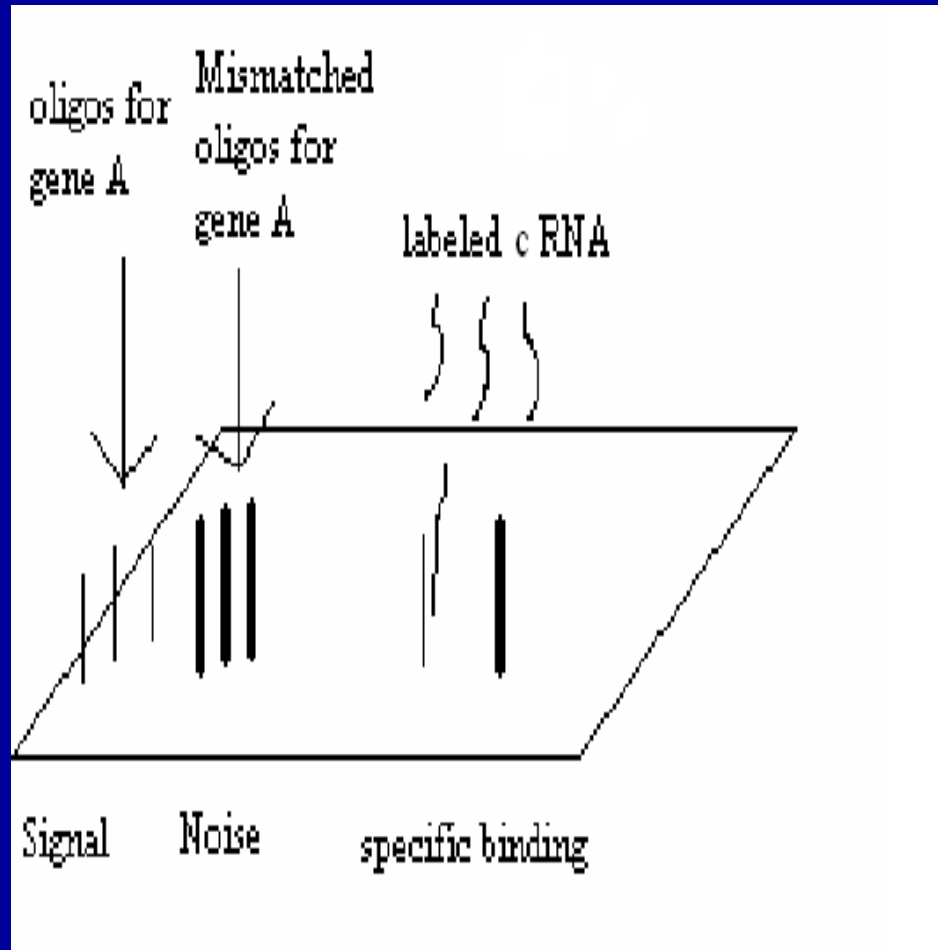
NT2 frataxin-RNAi neurons

Frataxin protein





Affymetrix Microarray format



- Oligonucleotides homologous to 12,599 genes on chip
- Most genes are of known function

Problems with microarray: Signal and Noise

- Weak Signal of frataxin-deficiency relative to 'Noise'.
- What makes the 'Noise'?:

  --inter-patient genetic variability

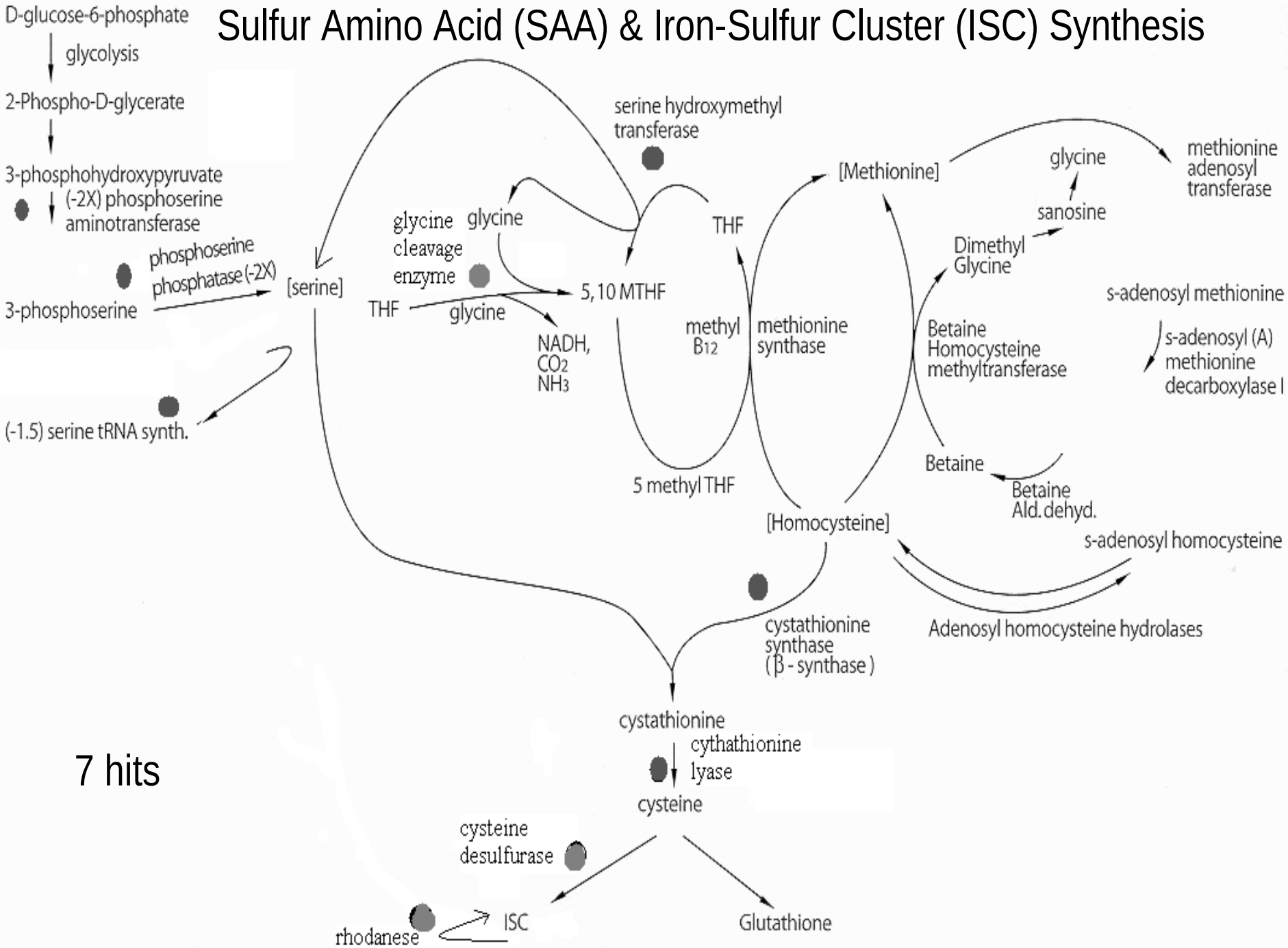
  --cell type variability

  --biochemical labeling variability

How to filter Signal from Noise:

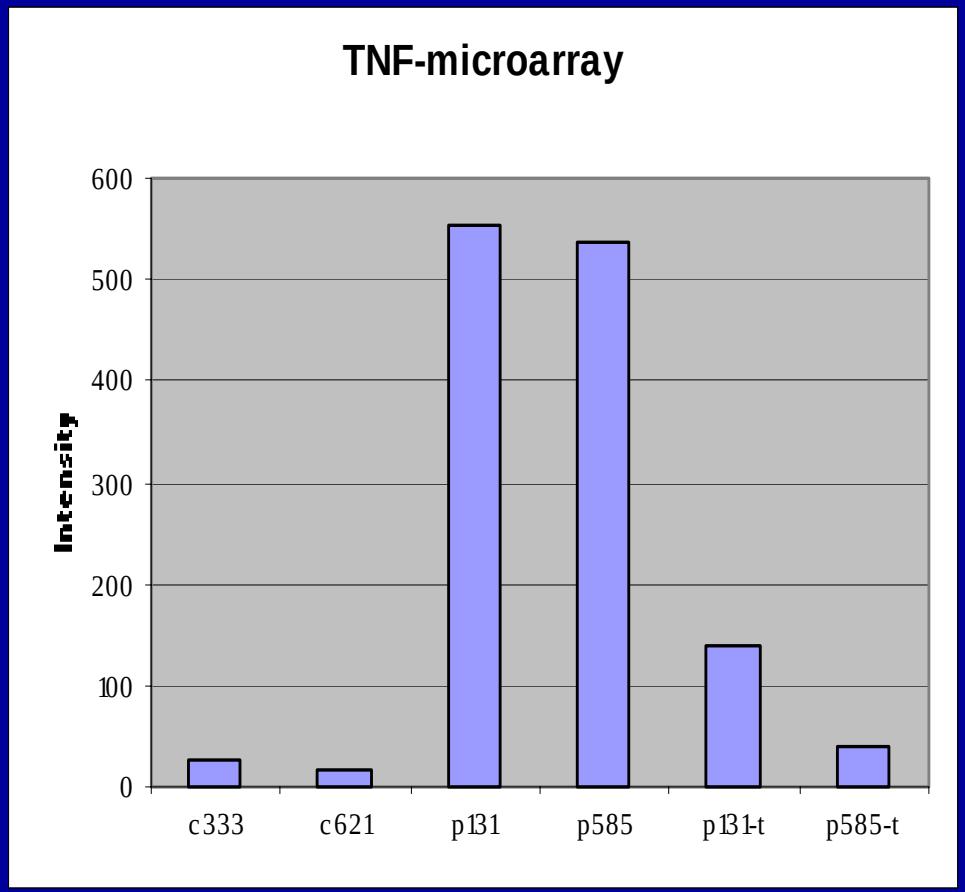
- Use many chips to determine Mean +/- standard deviation.
- Use cells from many Friedreich's ataxia patients.
- Compare results from different cell types.
- Use RT-PCR to confirm differential expression.

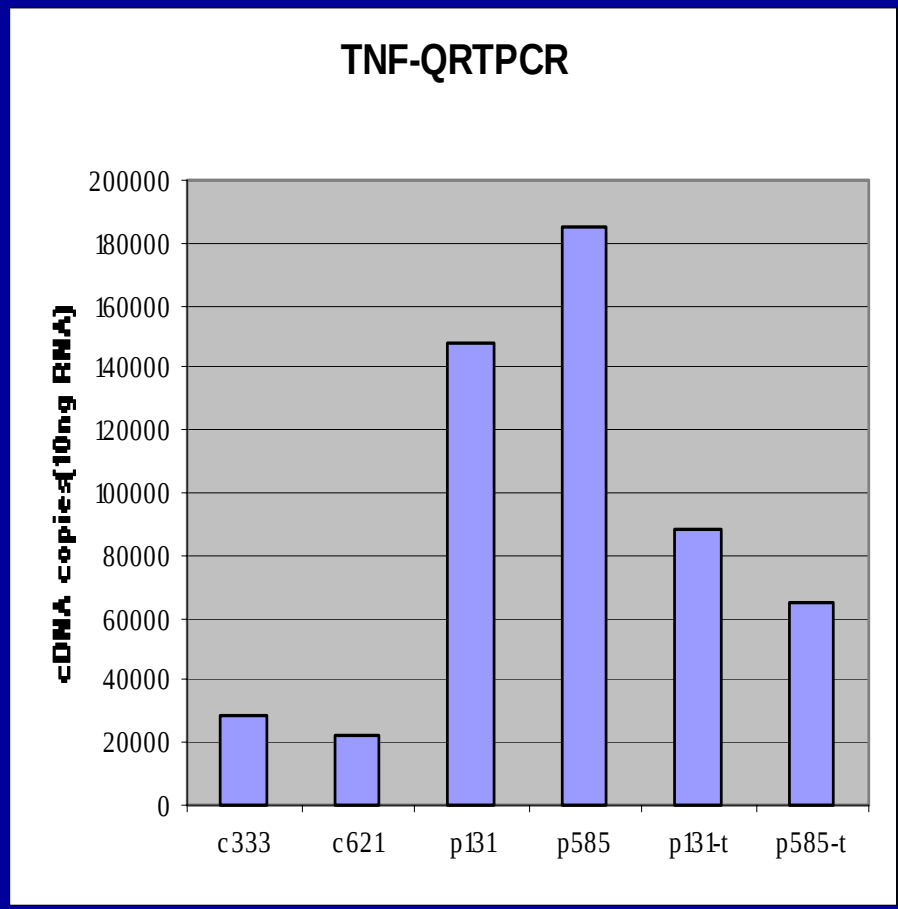
Sulfur Amino Acid (SAA) & Iron-Sulfur Cluster (ISC) Synthesis



7 hits

Confirmation of Microarray data by QRT-PCR:





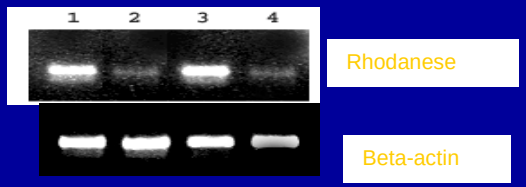
Summary of Microarray Data

- The two major frataxin-dependent alterations are:
  - A decrease in 7 SAA transcripts
  - An increase in apoptosis transcripts
- Of the 5 existing hypotheses, the score is 7:0:0:0:0 in favor of an SAA/ISC hypothesis

Confirmatory tests of an ISC/SAA hypothesis

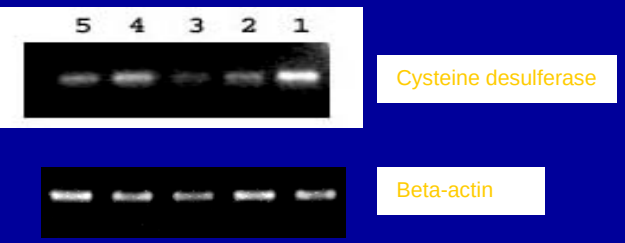
- Confirmation of decreased SAA/ISC transcripts by RT-PCR
- Measure a decrease in Sulfur Amino Acids concentration.
- Measure a decrease in Iron-Sulfur Cluster dependent enzymes.
- Identify a specific defect in ISC-biosynthetic pathway

Rhodanese & Cysteine Desulfurase mRNAs are deficient in mutants:



Rhodanese

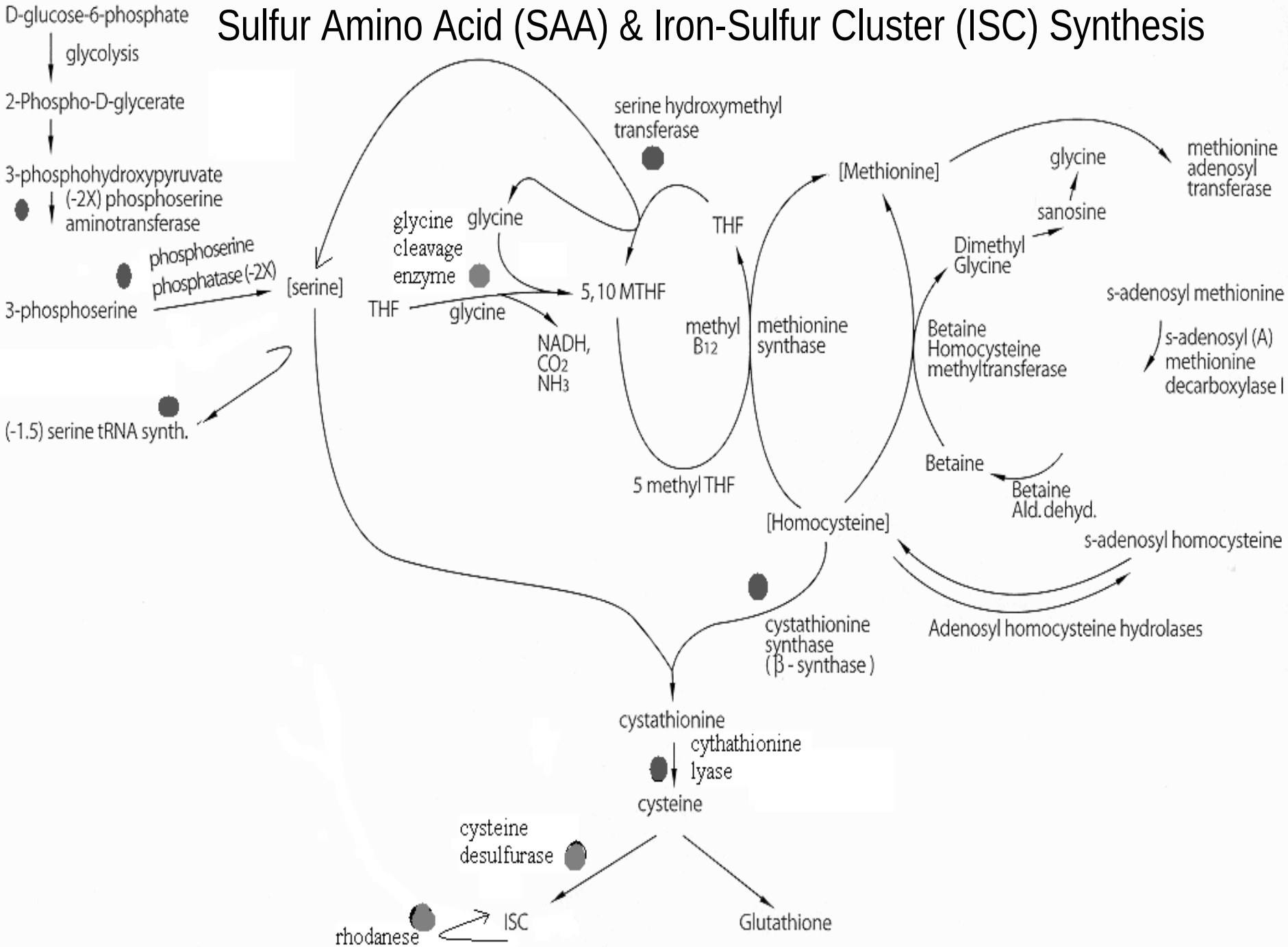
(Maintains mitochondrial FeS clusters)



Cysteine Desulfurase ISC-S

(provides Sulfur for FeS clusters)

Sulfur Amino Acid (SAA) & Iron-Sulfur Cluster (ISC) Synthesis



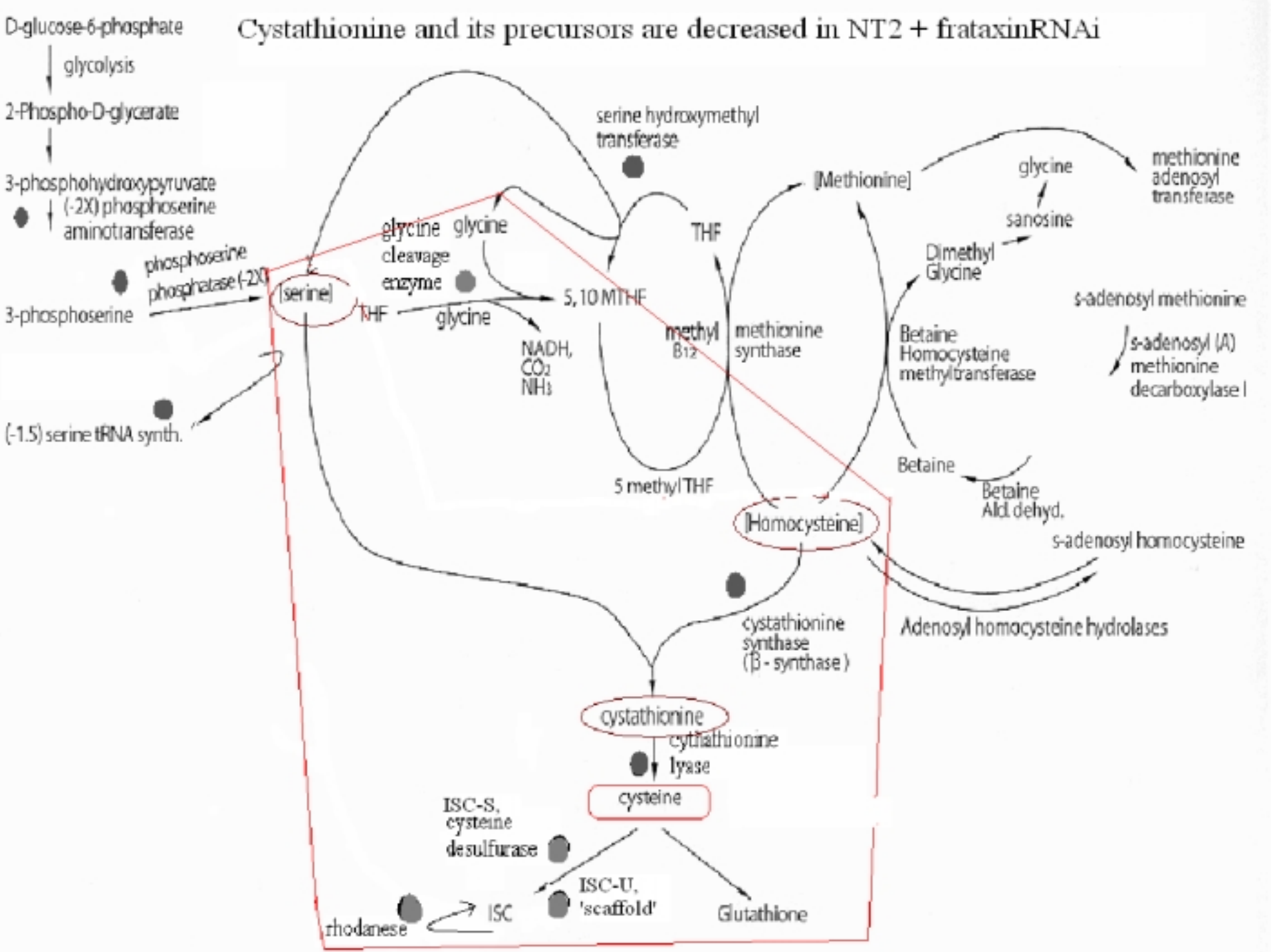
Sulfur Amino acid levels are decreased in FRDA cells:

In whole neural cell extracts inhibited by frataxin RNAi:

A Acid	Control-NT2	Frataxin-RNAi	P-value
Homocystine	**0.99±0.20**	**0.29±0.19**	**0.01***
Cystathionine	**16.16±6.99**	**10.62±7.23**	**0.02***
Serine	**109.7±9.0**	**88.71±4.91**	**0.03***
Ornithine	**6.65±2.25**	**4.80±2.11**	**0.02***

Lymphoblast mitochondria:			
Cysteine	2.63	0.96	0.03
Ornithine	6.0	2.6	0.03
Glycine	59	39	0.05

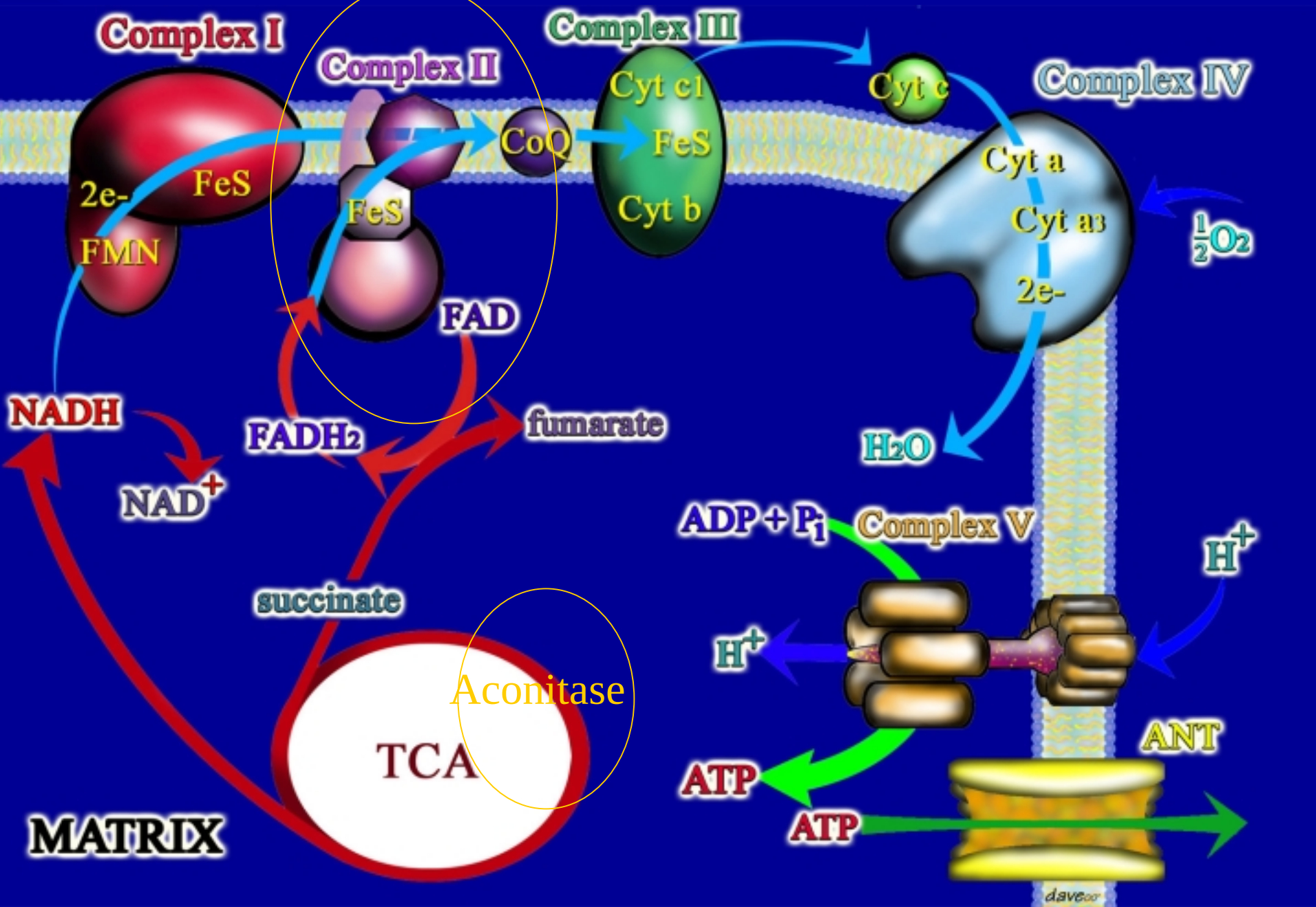
Cystathionine and its precursors are decreased in NT2 + frataxinRNAi



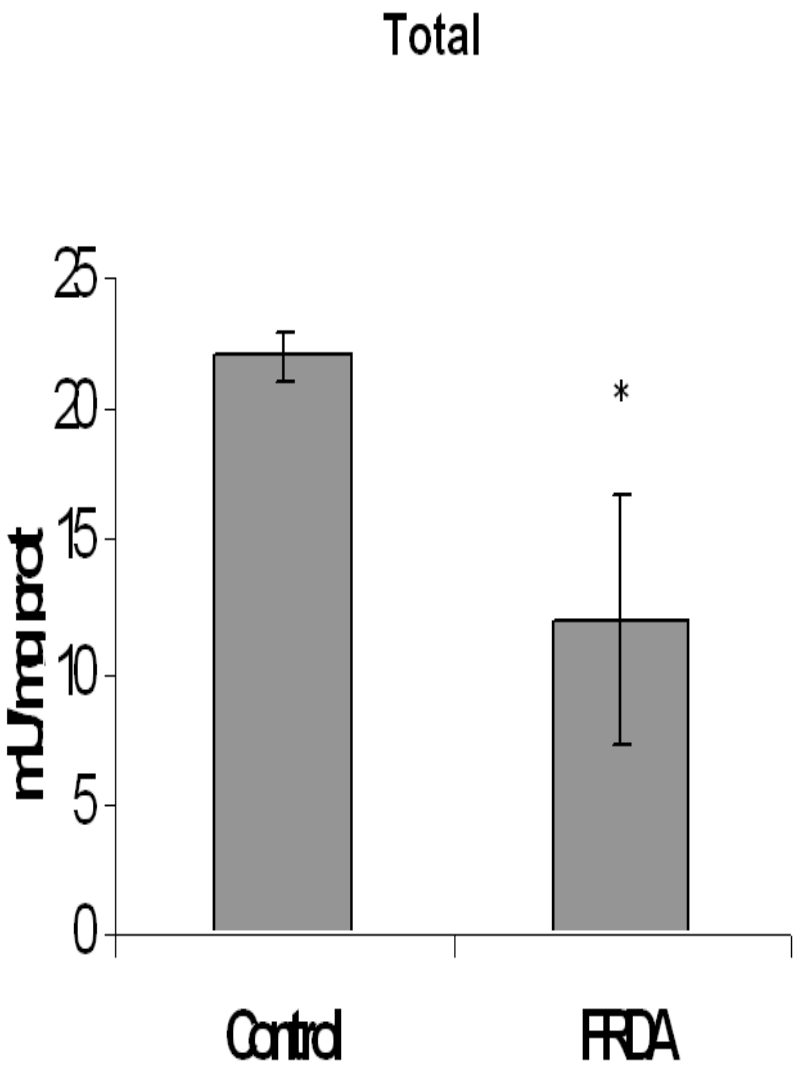
Decrease in ISC-requiring enzyme activity

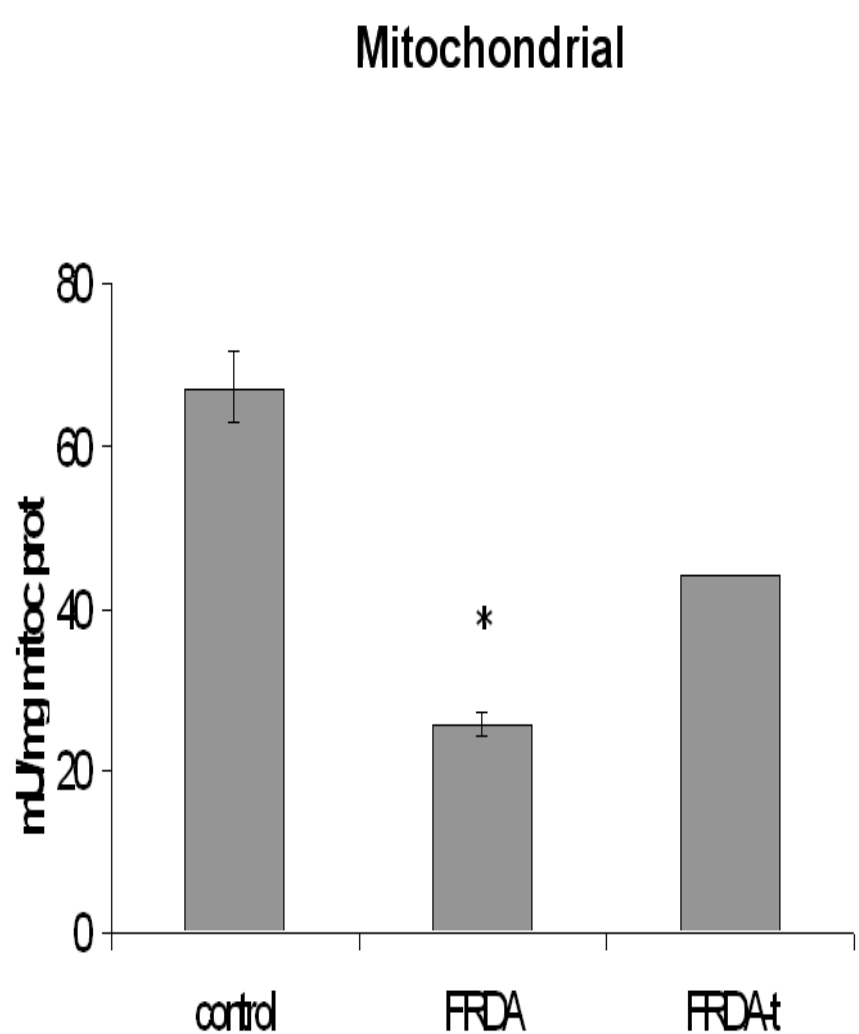
- Aconitase
- Succinate Dehydrogenase

Membrane potential is generated by TCA/Ox Phos

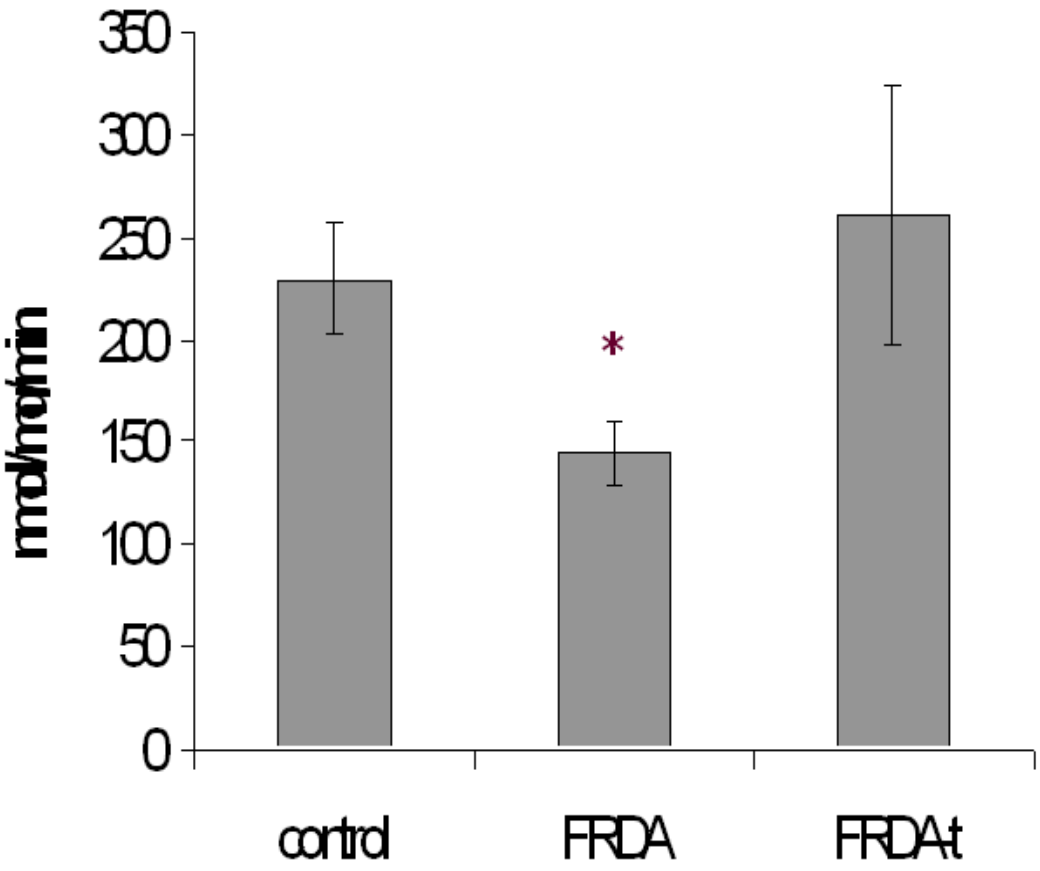


Aconitase activity



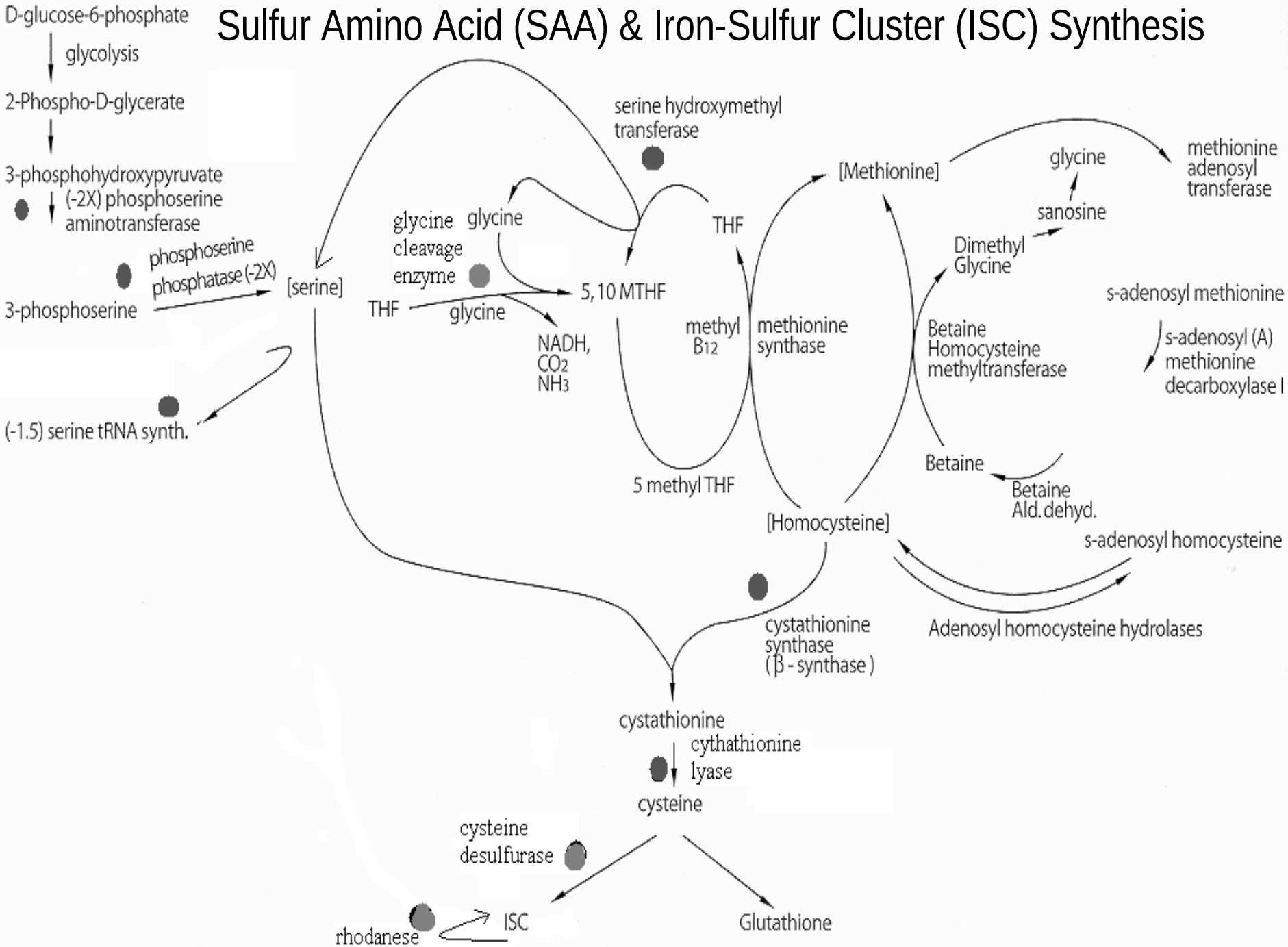


SDH activity

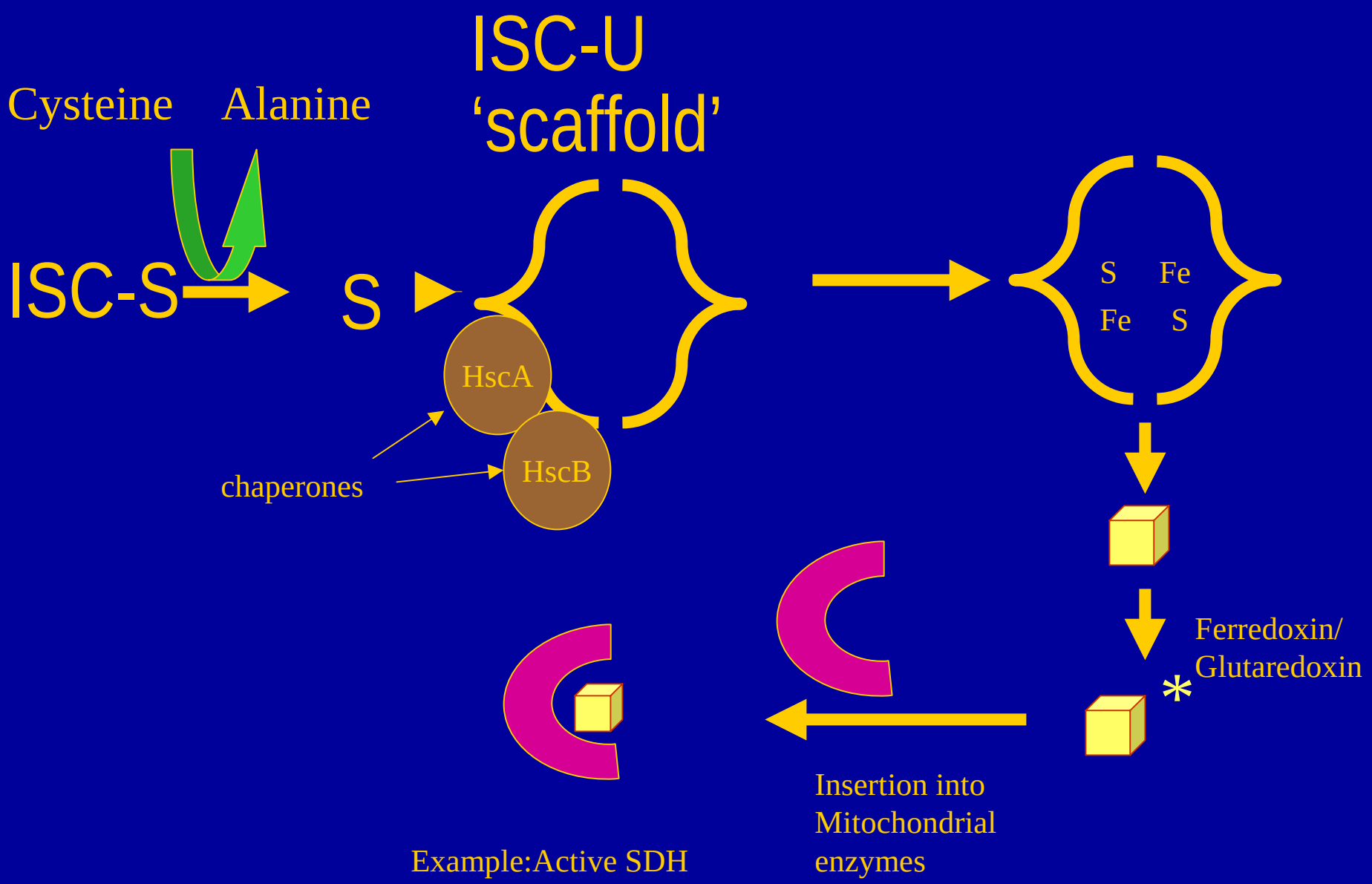


Is there a frataxin-specific effect on the Iron-Sulfur Cluster Biogenesis Machinery?

Sulfur Amino Acid (SAA) & Iron-Sulfur Cluster (ISC) Synthesis



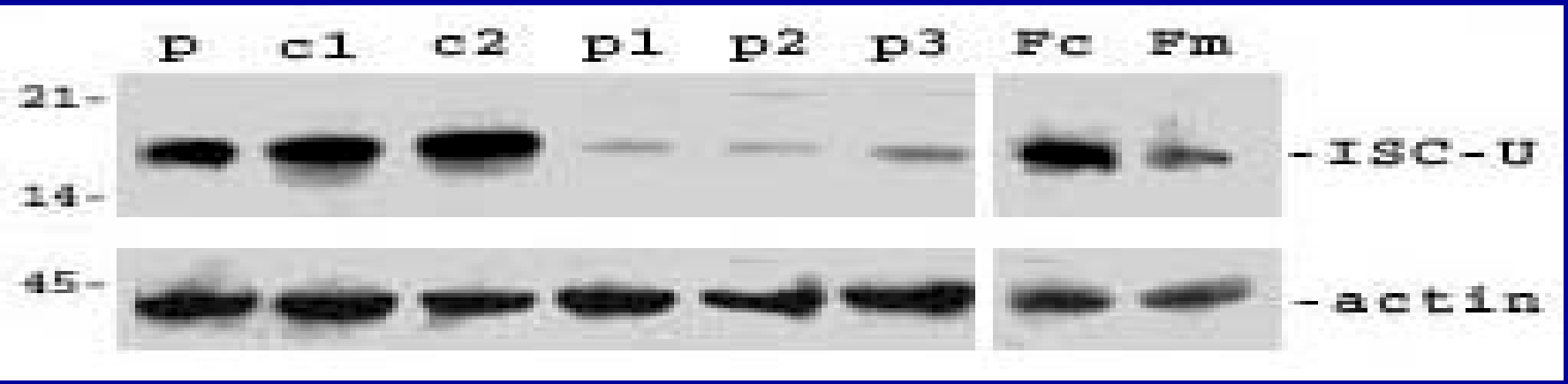
Model of ISC formation from bacteria:

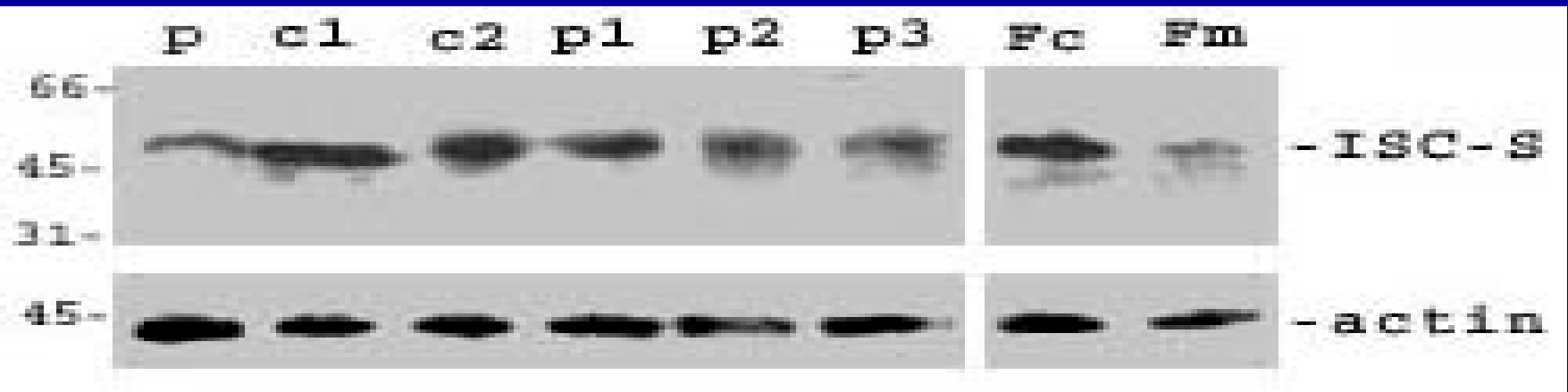


ISC-U protein is decreased in patient lymphoblasts

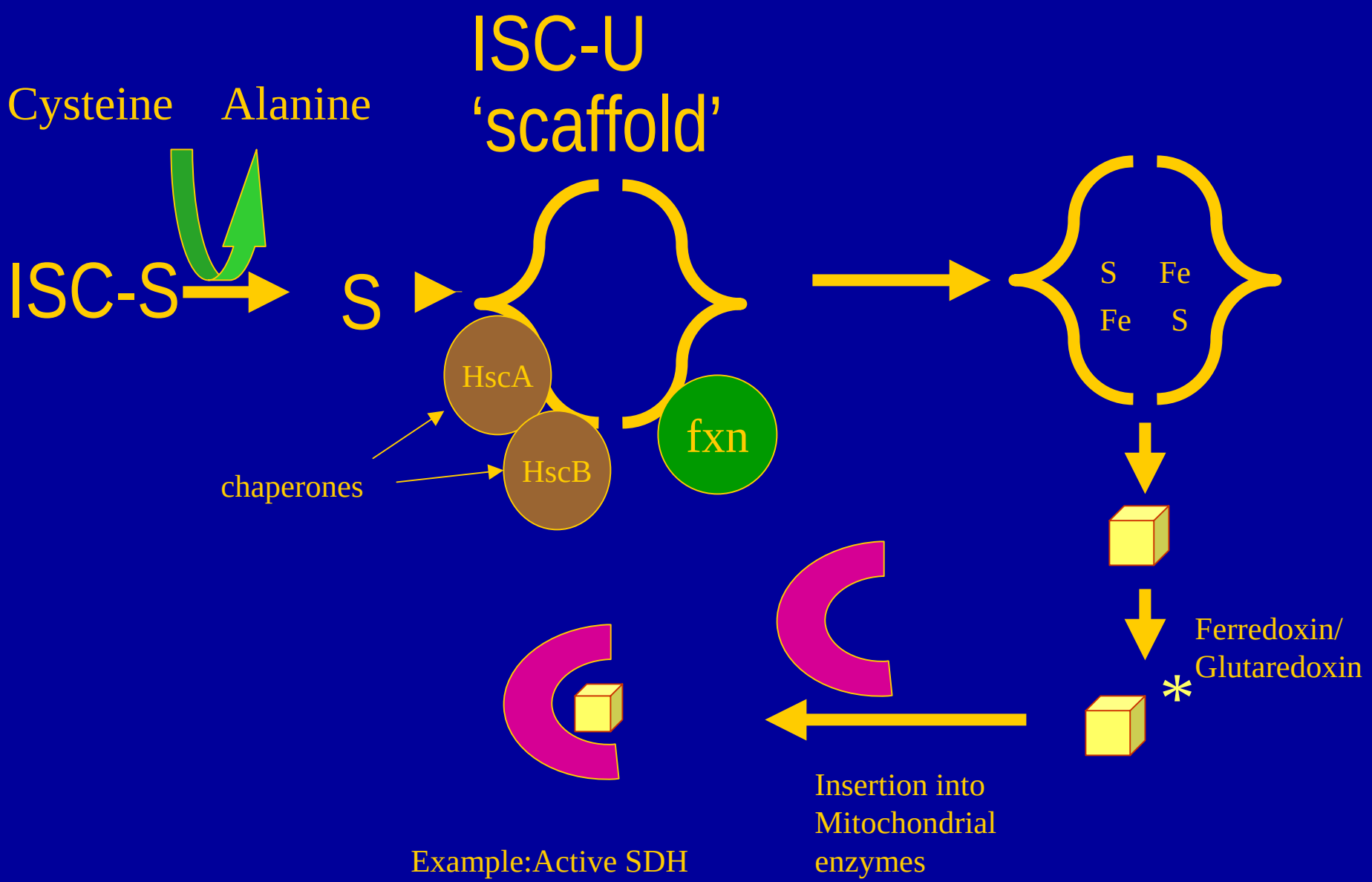
Lymphoblasts

Fibroblasts

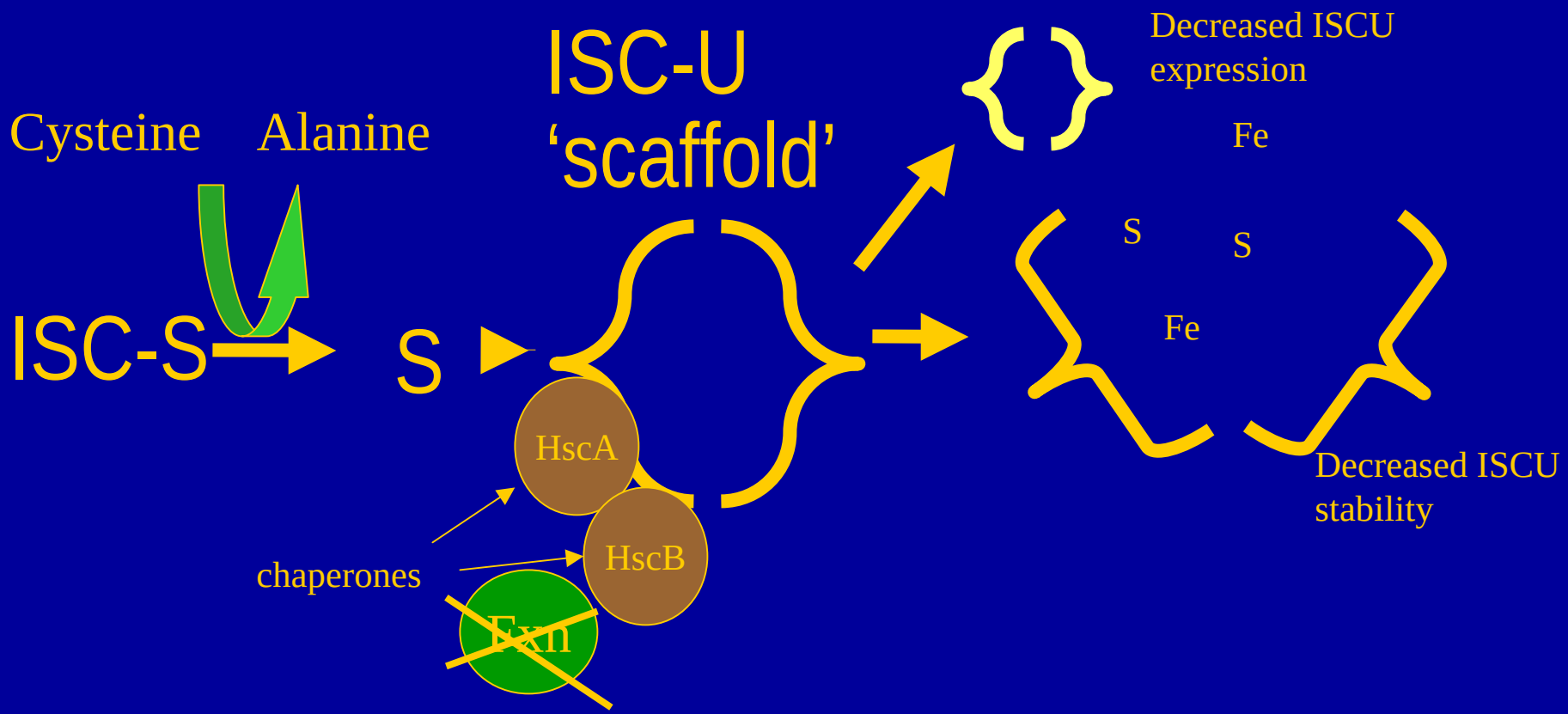




Frataxin may be required for ISC-U stability or expression:



Frataxin may be required for the expression, or stability of ISC-U

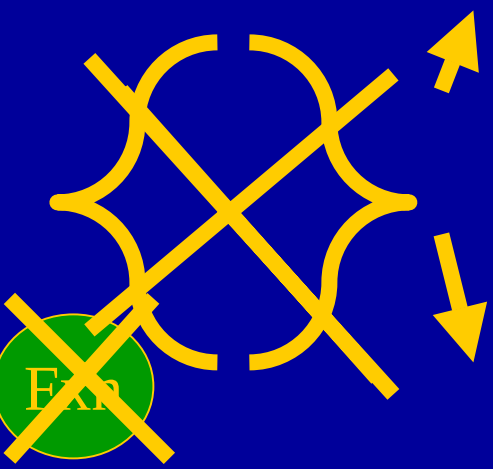


Confirmations of an SAA/ISC hypothesis

- 7:0:0:0:0 score by Microarray in favor of ISC/SAA hypothesis
- Confirmation of decreased SAA/ISC transcripts by PCR
- Decreases in [SAA]s in cell extracts and mitochondria
- Decreases in Iron-Sulfur Cluster dependent enzymes.
- Identified a specific defect in ISC-U expression in FRDA cells
- Appears to be a direct interaction between ISC-U and frataxin

How does a defect in FeS clusters cause cell death?

ISC-U
'scaffold'



Fe

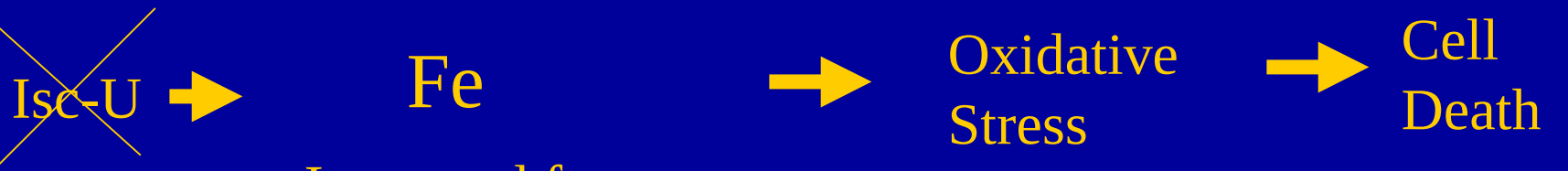
Increased free (toxic) iron → Oxidative Stress → Cell Death

(stress pathway)

Decreased FeS clusters → Decreased Mitochondrial Enzyme activity → Cell Death

(Cluster-bioenergetic pathway)

Potential routes to therapy in the stress pathway



Possible Rescue Agents	Iron chelator	Anti-oxidants	Anti-apoptotics
	Desferal, L1	Vitamin E Trolox (not Vit C.) Mito-Q Idebenone	No clinically available anti-apoptotic..yet

Fenton Chemistry contributes to oxidative stress

$$.O_2^- + Fe^{3+} \Rightarrow Fe^{2+} + O_2$$

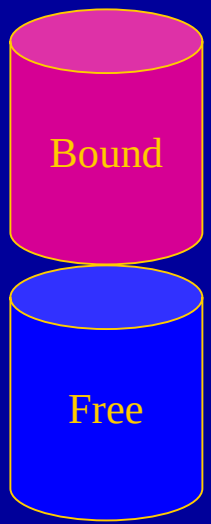
Superoxide
(produced by mitochondria)

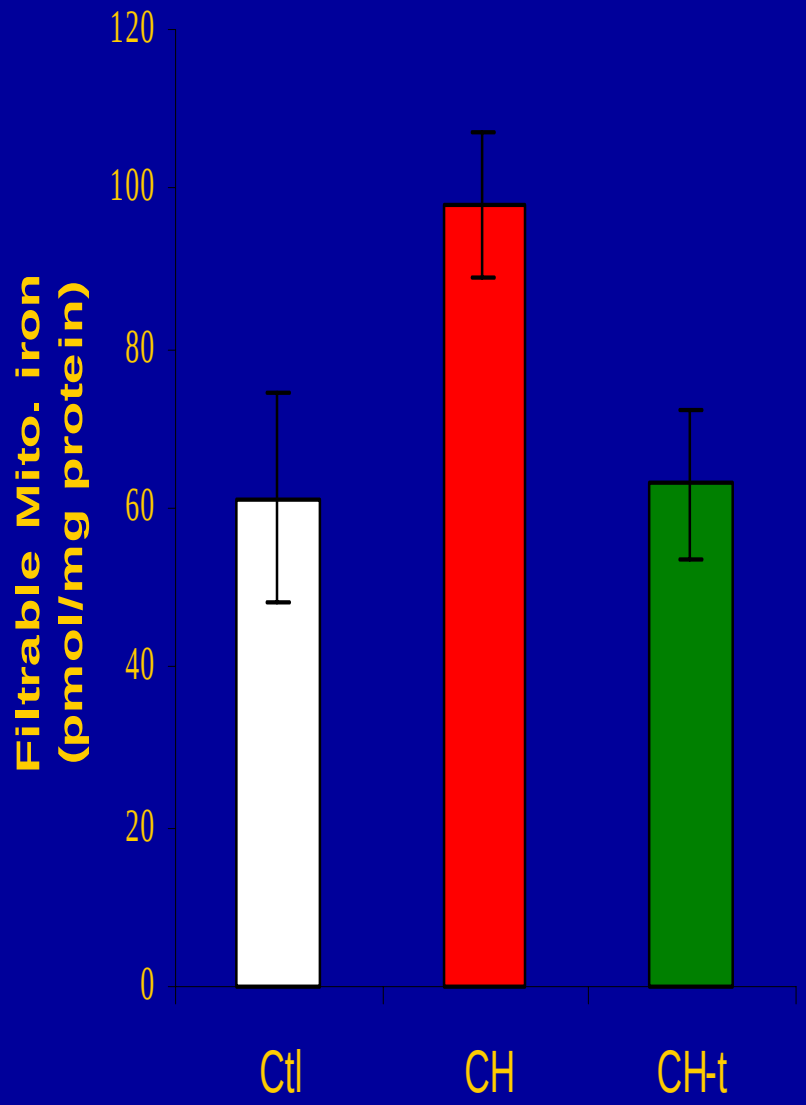
$$Fe^{2+} + H_2O_2 \Rightarrow .OH + HO^- + Fe^{3+}$$

Hydrogen Peroxide (toxic)

Hydroxyl Radical (toxic)

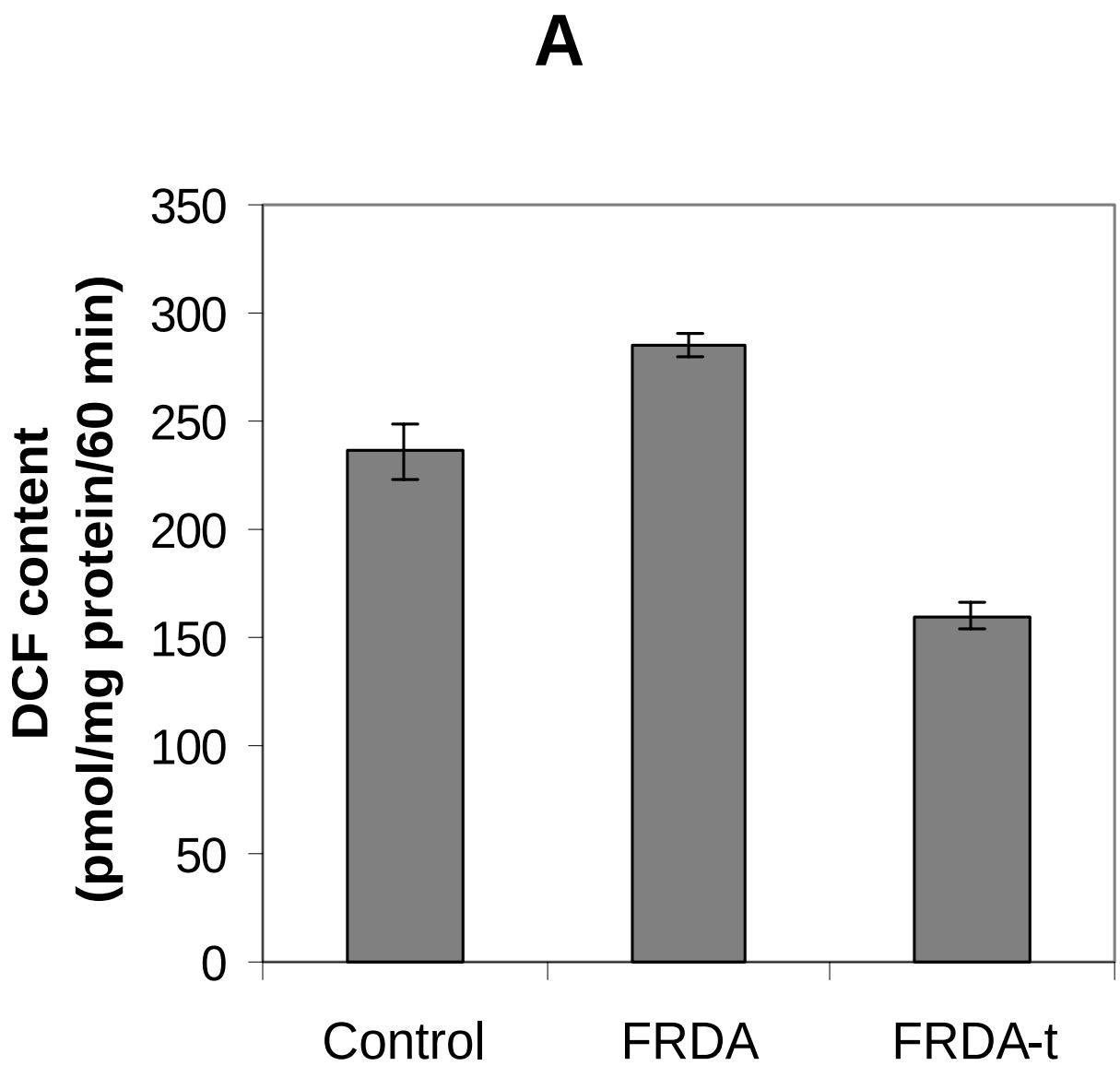
Frataxin levels affect free mitochondrial iron





Free Mito. Iron

Peroxides are increased in FRDA mitochondria



Potential routes to therapy in the stress pathway

~~Isc-U~~ → Fe → Oxidative Stress → Cell Death

Increased free (toxic) iron

Possible Rescue Agents	Iron chelator	Anti-oxidants	Anti-apoptotics
	Desferal, L1	Vitamin E Trolox (not Vit C.) Mito-Q Idebenone	No clinically available anti-apoptotic..yet

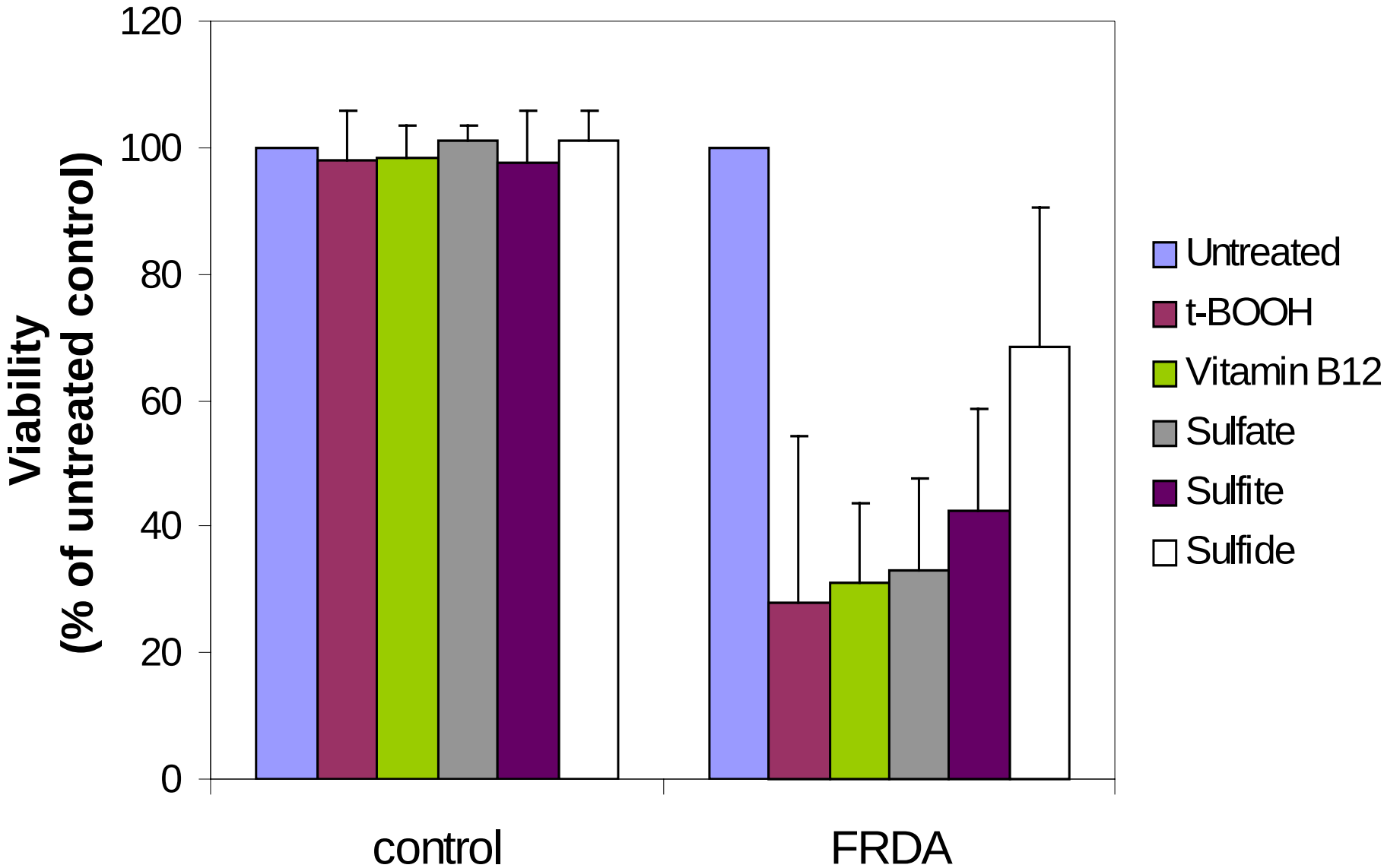
Potential routes to therapy in the Cluster-bioenergetic pathway

(Cluster-bioenergetic pathway)

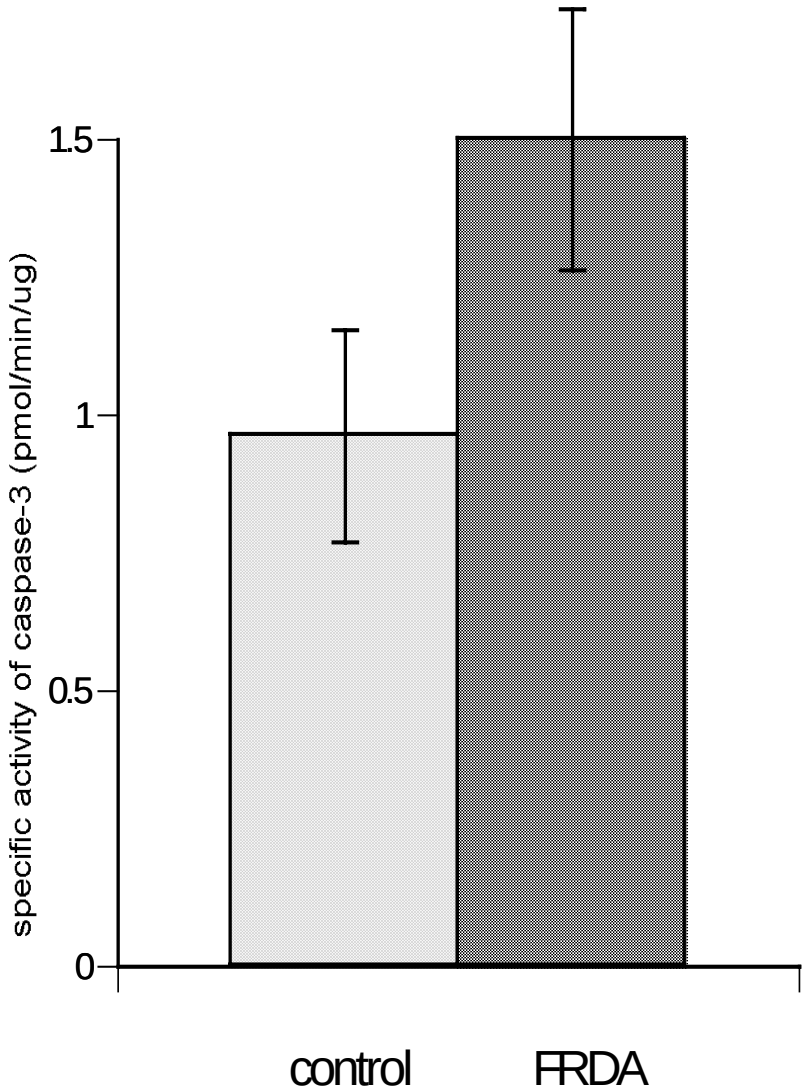
~~ISC-U~~ → Decreased FeS clusters → Decreased Mitochondrial Enzyme activity → Cell Death

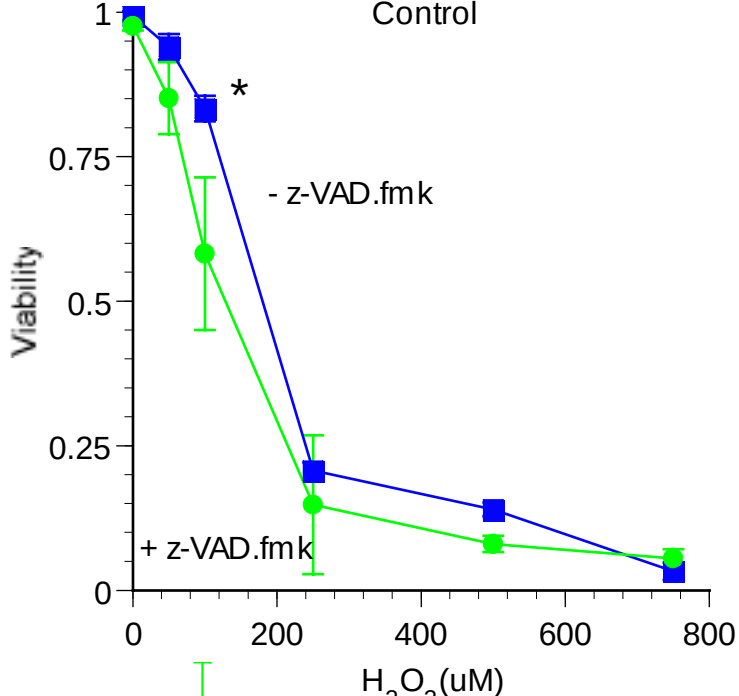
Potential Rescue Agents	Supplement Non-enzymatic FeS Cluster Synthesis	Supplement Mitochondrial Activity	Anti-apoptotics
Examples	Thiosulfate? Sulfur compounds?	Idebenone, Creatine	Not yet available

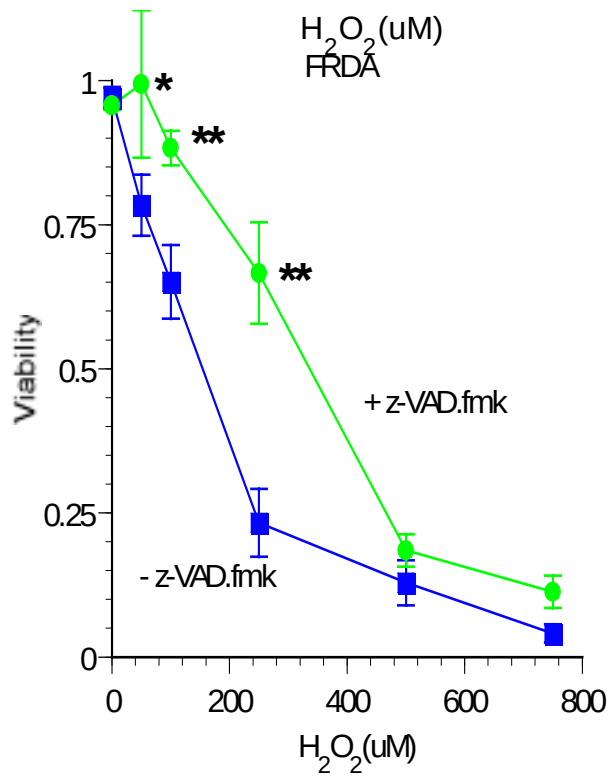
Sulfide rescues Friedreich's ataxia cells sensitivity to Oxidative Stress



ZVAD protects FRDA cells From apoptosis







Potential routes to therapy in the Cluster-bioenergetic pathway

(Cluster-bioenergetic pathway)

ISC-U → Decreased FeS clusters → Decreased Mitochondrial Enzyme activity → Cell Death

Potential Rescue Agents	Supplement Non-enzymatic FeS Cluster Synthesis	Supplement Mitochondrial Activity	Anti-apoptotics
Examples	Thiosulfate? Sulfur compounds?	Idebenone, Creatine	Not yet clinically available

Summary

1. Microarray only supports one of 5 hypotheses for frataxin.
2. There are multiple confirmations of FeS in human cells:
   - Decreased ISC/SAA transcripts by QRT-PCR
   - Decreased SAA levels
   - Defects in ISC-dependent enzymes
   - Defects in expression of ISC-U
3. There is evidence for increased apoptosis in human cells
4. Some therapy is available, and some others may soon be possible, but there is a very long way to go to specific, curative FRDA therapy

(We have a need for more cells from individuals with FRDA, and hope you could help)

- Guolin Tan — UC Davis- VM:Molecular Biosciences
- Eleonora Napoli — UC Davis & Universita di Padova
- Gino Cortopassi — UC Davis
- Franco Taroni — Istituto Neurologico ‘Carlo Besta’, Milan, Italy

- PHS/NIH
- FARA Foundation