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INSIGHTS FROM MODEL SYSTEMS
The Yeast Connection to Friedreich Ataxia

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There have been both genetic and biochemical advances in the understanding of inherited neurodegenerative diseases, such as Huntington disease (HD) and Friedreich ataxia (FA). In FA, these advances have come from the following two approaches: the mapping of the disease gene in humans, and work with a distantly related model organism, *Saccharomyces cerevisiae*. These two approaches converged, with the study of the human gene leading to the yeast homologue and the study of the yeast mutant phenotypes leading to the human homologue and its associated disease.

FA has an estimated prevalence of 1/50,000 in European populations, making it the most common inherited ataxia. The neurologic symptoms, which start during adolescence, include gait and limb ataxia, lower limb areflexia and pyramidal weakness, loss of proprioception, and dysarthria. Most patients develop hypertrophic cardiomyopathy and skeletal abnormalities, and some become diabetic (Durr et al. 1996). These symptoms progress with age, such that most patients become wheelchair-bound by their late twenties and die by their mid-thirties—most commonly of congestive heart failure.

Genetically, FA belongs to a class of neurodegenerative disorders in which the underlying gene, *FRDA1,* carries an unstable trinucleotide-repeat sequence. At least eight other members of this class have been identified, including HD and many types of spinocerebellar ataxia. However, key genetic features separate FA from the other trinucleotide-repeat disorders. First, the sequence of the trinucleotide repeat in the *FRDA1* gene is GAA (Campuzano et al. 1996), whereas a CAG repeat occurs in the other trinucleotide-associated ataxias, and other repeats (CTG or CGG) are seen in other trinucleotide diseases. Second, the GAA repeat of *FRDA1* is located in the first intron and is therefore noncoding, whereas the CAG repeat in HD and the spinocerebellar ataxias always occurs within an exon and encodes glutamine (Campuzano et al. 1996). The third difference is that FA is inherited in a recessive manner, and multiple lines of evidence suggest that loss of function leads to the disease. In contrast, in the other trinucleotide-repeat disorders, whether the repeat occurs in an expressed DNA sequence (Paulson 1999 [in this issue]) or in a 3′ untranslated sequence (Timchenko 1999 [in this issue]), the mutation is inherited in a dominant manner, and it is a gain of function of the affected protein or RNA that perturbs cell physiology.

The severity of the disease correlates with decreased *FRDA1* expression and with the length of the hyperexpansive repeat. Normally, this gene, which encodes the protein frataxin, contains <39 GAA repeats, but in patients with FA, this locus contains 66–1,700 repeat units. This hyperexpansion results in marked decreases in frataxin mRNA levels, thought to result from the formation of an unusual non-β DNA structure inhibiting transcription (Bidichandani et al. 1998; Sinden 1999 [in this issue]). More than 95% of patients with FA are homozygous for the GAA hyperexpansion, although the alleles are polymorphic in the number of GAA repeats. Studies have shown a correlation between the length of the GAA expansion on the smaller allele and severity of disease (Durr et al. 1996). An inverse correlation between GAA expansion size and frataxin protein levels has been observed in lymphoblast cell lines from patients with FA (Campuzano et al. 1996). Together, these findings suggest that lack of frataxin protein in critical tissues leads to FA. The remaining 5% of patients with FA are compound heterozygotes for the GAA expansion on one allele and carry point mutations within *FRDA1* on the other allele.

The most common disease-causing point mutation in frataxin is I154F (numbering based on the initiator methionine of the predicted open reading frame [ORF]), prevalent in some southern Italian families. Those individuals carrying this missense mutation on one allele, together with the hyperexpansion on the other allele, are indistinguishable in disease severity when compared

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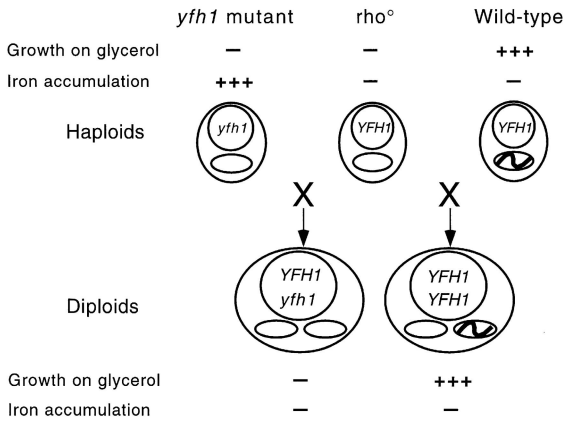
Ironclad Evidence

In yeast cells, as in human cells, nuclear and mitochondrial genes are required, for mitochondria to function. Yeast mtDNA encode eight polypeptides (compared with 13 open reading frames in human mtDNA), plus ribosomal and transfer RNA. The major mitochondrial functions, oxidative phosphorylation and oxidative metabolism of fatty acids, clearly are essential in metazoans, but yeast cells completely lacking mtDNA can be grown indefinitely, so long as fermentable carbon sources, such as glucose, are provided in their medium. For this reason, nuclear or mitochondrial mutations that affect oxidative phosphorylation can readily be identified as mutant yeast cells that fail to grow on a nonfermentable carbon source, such as glycerol. Such yeast strains are termed "pet" mutants, because their growth, even on glucose, is slow, leading to small ("petite") colonies. When pet mutants arise from mutations in mtDNA, they may be described as either ρ^- or ρ°, depending on whether the mtDNA has undergone large deletions or has been lost from the cell entirely. The figure below depicts the strategy we applied to show that one pet yeast strain, mutant for a nucleus-encoded gene, *Yfh1*, is ρ^- or ρ° as a consequence of this mutation. *Yfh1* encodes a homologue of the human frataxin protein, which is implicated in Friedreich ataxia. We crossed the *yfh1* mutant with a ρ° strain, in which the nuclear genome was wild type. The resulting diploid yeast cell, like the *yfh1* $^-$ parental strain, could not grow on the glycerol carbon source, indicating that mtDNA in *yfh1*$^-$ cannot restore mitochondrial function, even when the Yfh1p protein is available from the other parental strain.



A link between iron and mtDNA damage was suggested, from the independent cloning of *YFH1* as a high-copy suppressor of a mutant yeast that was defective in its use of intracellular iron. Loss of *YFH1* function leads to mtDNA damage and to mitochondrial accumulation of iron. Another relevant gene, *SSQ1,* was identified in a screen for yeast mutants exhibiting altered iron homeostasis. Like *yfh1* mutants, *ssq1* cells accumulate iron in mitochondria and sustain mitochondrial DNA damage. Because *SSQ1* encodes a putative mitochondrial Hsp70, we looked for evidence that chaperone defects affect Yfh1p maturation, and we observed that Yfh1p processing in mitochondria was compromised in *ssq1* yeast. We suggest that the human frataxin protein plays a similar role in intracellular iron trafficking and that other human genes, analogous to *SSQ1,* may be relevant to the pathogenesis of certain other disorders related to Friedreich ataxia.

with homozygous relatives who carry the GAA triplet expansion on both alleles (Campuzano et al. 1996). Another missense mutation in frataxin, G130V, compounded with a hyperexpansive allele, is associated with a milder and more slowly progressive disease course (Bidichandani et al. 1997). Of note is that disease-causing point mutations in frataxin occur in residues conserved with the homologous yeast protein. The precise biochemical function that is disrupted is unknown, but it may relate to iron metabolism.

Phenotypic variants of FA, which nonetheless are caused by mutations at the *FRDA1* locus, include FA with retained lower limb reflexes, late-onset FA, and the more slowly progressing Acadian FA variant (Palau et al. 1995). These less-severe variants of FA often occur in individuals carrying intermediate lengths of the GAA repeat. Because of this proportionate loss of expression of *FRDA1* as the GAA trinucleotide expands, the disease mechanism in FA is more analogous to that of fragile X–linked mental retardation (FXMR) than to the other repeat-associated ataxias. However, the analogy to FXMR is also imperfect, because, in that disorder, loss of gene expression is associated with CpG methylation in long CGG tracts in the 5′ untranslated region of the first exon of FMR1. Such a mechanism is incompatible with the structure of the intronic GAA repeats in *FRDA1*.

The Yeast Connection

At the time of its identification, the deduced 210–amino acid sequence of frataxin revealed no clue to its function, other than the presence of an N-terminal sequence that suggested mitochondrial localization (Campuzano et al. 1996). The frataxin ORF is homologous to ORFs from *Caenorhabditis elegans* and *S. cerevisiae*. A homologue is also present in a gram-negative bacterial species, whose genomic structure suggests that it is related to the predecessors of eukaryotic mitochondria. Thus, the frataxin gene may have existed first within the ancestral mtDNA of eukaryotes and then moved to the nuclear genome at some time early in eukaryotic evolution.

The connection of FA with mitochondrial function was significantly strengthened by the study of the yeast frataxin homologue encoded by *YFH1*. The encoded protein was localized to yeast mitochondria by immunofluorescence microscopy. Furthermore, disruption of this gene caused the yeast to be unable to grow on nonfermentable carbon sources (e.g., glycerol plus ethanol) and to grow poorly on fermentable carbon sources (e.g., glucose). The *yfh1* mutant strains thus display a "petite" phenotype (see sidebar) and are unable to carry out normal oxidative phosphorylation (Babcock et al. 1997; Foury and Cazzalini 1997; Koutnikova et al. 1997; Wil-

son and Roof 1997), at least in part because of mtDNA damage.

A major insight into FA came when seemingly unrelated studies on metal metabolism in yeast suggested the involvement of iron. Iron transport into yeast is mediated by ferric reductases of the plasma membrane (Fre1p and Fre2p), which reduce iron to its ferrous form before it is brought into the cell through Ftr1p, an iron permease, and its associated multicopper oxidase, Fet3p (Askwith and Kaplan 1998). All of these genes are under the control of Aft1p, an iron-sensitive transcriptional factor. When yeast lack iron, Aft1p activates transcription of the iron-uptake components, and conversely, when iron is plentiful, Aft1p-dependent transcription ceases. Although this cellular import pathway is well understood, little is known about the trafficking of iron once it enters the cell. Therefore, Li and Kaplan (1996) sought mutants that were defective in intracellular iron usage. *YFH1* was identified as a high copy-number suppressor of one such mutant, thereby linking the yeast frataxin homologue to iron (Babcock et al. 1997).

Cells lacking *YFH1* exhibit constitutive activity of the high-affinity iron uptake system. Cytosolic iron is diminished in *yfh1* cells (Knight et al. 1998), whereas mitochondrial iron is ⩾10-fold higher (Babcock et al. 1997; Foury and Cazzalini 1997). This suggests that lack of Yfh1p causes iron accumulation in mitochondria and breakdown of cellular iron homeostasis. The exact cause of the mitochondrial iron accumulation is not known; however, a role for Yfh1p in efflux of iron from the mitochondria has been suggested (Askwith and Kaplan 1998).

Iron and FA

With new insight gained from yeast genetic studies that suggest FA may be associated with altered cellular iron distrubution, it is possible, in hindsight, to find supportive evidence from earlier studies in the biochemical and clinical literature. One such study evaluated the fate of radioactive iron citrate administered to patients with FA, compared with individuals with Hallervorden-Spatz disease (Szanto and Gallyas 1966). The patients with FA exhibited more-rapid turnover of iron in plasma and in red blood cells, suggesting a decrease in red-cell survival. Plasma iron concentration, plasma iron binding capacity, and urinary iron were not affected. Measurements of levels of hemoglobin, bilirubin, lactate dehydrogenase, and reticulocyte counts were not reported. A subsequent study has confirmed that patients with FA exhibit normal levels of serum iron and ferritin (Wilson et al. 1998). However, these measurements reflect red-cell iron metabolism, which may be regulated by specifically dedicated feedback loops, as compared with other tissues.

Despite the ubiquitous expression of frataxin in all tissues, the primary effects of FA are seen in the central nervous system, specifically in large sensory neurons and dorsal root ganglia. The heart is also targeted, and the resulting cardiomyopathy limits life expectancy (Koutnikova et al. 1997). The reasons for this pattern of tissue sensitivity are not known, although they may relate to the metabolic rate and abundance of mitochondria in these tissues (Koutnikova et al. 1997). Postmortem examination of hearts from patients with FA have shown zones of cardiac muscle degeneration, with many iron deposits within myocardial fibers (Lamarche et al. 1980). Electron microscopy of myocardial fibers showed electron-dense deposits, surrounded by membranes and associated with damaged mitochondria. These deposits were termed "lipofuscin"—an ill-defined autofluorescent aggregate, containing polyunsaturated lipids and proteins. Although these deposits were not analyzed for iron, it is tempting to speculate that it might be present.

Iron is a cofactor for numerous heme and Fe-S proteins of mitochondria. In the electron transport chains of yeast and mammals, for example, complex II (succinate: ubiquinone oxidoreductase) contains Fe-S clusters as prosthetic groups, whereas complex III (bc_1 complex) contains both Fe-S in the Rieske iron-sulfur protein and heme in the form of cytochromes b and c_1, and complex IV (cytochrome c oxidase) contains heme as cytochromes a and a_3. Also present in mitochondria is aconitase, an Fe-S enzyme of the tricarboxylic acid cycle. In endomyocardial biopsied tissue from two patients with FA, activity levels of complexes II and III were found to be in the low to normal range. Of the enzymes analyzed, aconitase activity exhibited a marked decrease, relative to that of normal controls. Low enzyme activities were noted only in the diseased heart tissue and not in the unaffected tissues that included skeletal muscle, lymphocytes, or skin fibroblasts (Rotig et al. 1997). Similarly, in the *yfh1* yeast mutants, the activities of complexes II, III, and IV, as well as aconitase, were reduced. Overall, the *yfh*1 yeast mutant and disease tissue from patients with FA exhibit extraordinary similarities. Iron accumulates in mitochondria (in the yeast mutant and perhaps in the human disease tissue) in vast excess, and yet, paradoxically, iron proteins are deficient. The effects are more severe in the yeast than in the FA tissue, perhaps reflecting the difference between reduced expression of *FRDA1* in humans versus *YFH1* deletion in yeast.

The Importance of Mitochondrial Chaperones for Iron Homeostasis

In a screen for mutations affecting iron metabolism in yeast, another mutant that accumulates iron in the mitochondria was isolated (Knight et al. 1998). The defect in this case was in a nuclear gene encoding a mi-

tochondrial heat-shock protein 70 (Hsp70). In yeast, there are two mitochondrial Hsp70 (homologous to DnaK in bacteria), encoded by the genes *SSC1* and *SSQ1*. These proteins belong to the class of Hsp70 chaperones that facilitate the importing, processing, and folding of proteins in various organelles. Most proteins destined for the mitochondria are encoded in the nucleus and translated on cytoplasmic ribosomes. Hydrophobic regions of proteins interact with Ssc1p in the mitochondrial matrix during import (Kang et al. 1990). This chaperone acts in association with at least 2 other proteins, Mdj1p (a homologue of the bacterial protein DnaJ) and Mge1p (a GrpE homologue). These three proteins use energy from ATP hydrolysis, to ensure that the incoming protein folds correctly (Horst et al. 1997). For a subset of proteins, a second maturation system is required, which consists of Hsp60 (GroEL) and Hsp10 (GroES). This complex traps the nonfolded protein and allows the protein to fold into its native form. The protein is then released, after ATP hydrolysis. The released protein may achieve its native conformation immediately, or it may require additional rounds of interaction with the chaperone before reaching its final state (Martin 1997).

The Hsp70 gene, *SSC1,* is essential and abundantly expressed in yeast. The second mitochondrial Hsp70, *SSQ1,* originally was cloned as a homologue of *SSC1* (Schilke et al. 1996). *SSQ1* was found to be nonessential and expressed at low levels, when compared with *SSC1*. It was also found to play a role in maintaining mtDNA (Schilke et al. 1996). *SSQ1* was independently cloned from a screen of yeast mutants with defective cellular iron homeostasis (Knight et al. 1998). Like *yfh1* mutants, *ssq1* cells exhibit increased cellular iron uptake, iron diversion to the mitochondria, and accumulation of mtDNA damage (Knight et al. 1998). This phenotypic similarity between *yfh1* and *ssq1* mutations prompted investigations to determine whether Ssq1p is required for Yfh1p import and processing in mitochondria.

In wild-type cells, Yfh1p is imported as a preprotein and is subjected to two sequential processing cleavages at the N-terminus (Knight et al. 1998). The first cleavage removes ~2 kDa, and the second cleavage removes another 4 kDa. In the *ssq1* mutant, the second cleavage is impaired kinetically. The mechanism by which Ssq1p participates in the second processing step of Yfh1p is not known, but it may hold Yfh1p in a conformation that favors processing. Alternatively, Ssq1p may be required for the correct folding of Yfh1p, for its insertion into a multiprotein complex.

SSQ1 appears to be involved in the processing of Yfh1p within mitochondria. By contrast, import and proteolytic processing of Rieske iron-sulfur protein, cytochrome b_2, and cytochrome c_1, all components of Complex III, do not require Ssq1p. The import and processing of a noniron protein, prePut2, an enzyme of proline biosynthesis, also proceeds normally in the *ssq1* mutant strain (Schilke et al. 1996; Knight et al. 1998). However, it would be surprising that a specific chaperone is required for the processing of a single protein, and so it seems likely that other substrates for the Ssq1p chaperone will be identified.

Mechanisms of Cellular Toxicity in FA

The biochemical data from the yeast mutants suggest several mechanisms by which cellular damage might occur in patients with FA. According to one scenario, the accumulation of large amounts of iron within mitochondria leads to production of reactive oxygen species via the iron-catalyzed Fenton reaction (Babcock et al. 1997; Foury and Cazzalini 1997). The highly reactive hydroxyl radicals produced by this chemistry then react and damage any molecules in their vicinity, including DNA, proteins, and lipids. Evidence for this hypothesis comes from the observed hypersensitivity of *yfh1* cells to environmental oxidants. The situation is likely to be much more complex, however. Although the mitochondria of *yfh1* and *ssq1* yeast mutants are full of iron, mitochondrial iron proteins lack activity. The iron, though plentiful, appears to be biologically unavailable, either because it cannot be incorporated into heme or iron-sulfur clusters or because the iron proteins are destabilized. The resulting iron protein deficiency likely results in problems with the numerous functions subserved by these proteins, such as oxidative phosphorylation and amino acid metabolism.

These two mechanisms of cellular toxicity—oxidative stress and iron protein deficiency—are not independent of each other. Iron-sulfur proteins, such as aconitase, are known to be exquisitely sensitive to superoxide, which is capable of attacking and destroying iron-sulfur clusters. Thus, oxidant stress might directly cause iron-sulfur protein deficiency. The release of the protein-bound iron could in turn exacerbate the iron overload already existing. An important feature of the yeast mutants exhibiting iron overload and iron protein deficiency is that these effects occur primarily within mitochondria. Cytosolic iron levels remain to be relatively decreased. This intracellular compartmentalization of iron may be critical in explaining the mechanisms and targets of cell damage. Interestingly, yeast with mutations in the *SSQ1* gene were also isolated as suppressors of *sod1* mutant yeast (Strain et al. 1998). *SOD1,* encoding the cytosolic copper-zinc superoxide dismutase, is responsible for protection of the cytosolic compartment from excess superoxide by dismutation of superoxide to hydrogen peroxide and oxygen. This observation suggests that lack of function of the mitochondrial chaperone, *SSQ1,* partially *relieves* cytosolic oxidative stress.

Other mechanisms of cellular toxicity, suggested by

examination of the yeast model, may be relevant to the pathogenesis of FA. Hsp70 proteins are required, to ensure correct folding of a large number of proteins. Ssq1p may be involved in the correct folding of a subset of mitochondrial proteins. Thus, the loss of Ssq1p could lead to toxic protein aggregates similar to those observed in other neurodegenerative diseases, such as Huntington and Parkinson diseases. Electron micrographs of the *ssq1* yeast mutant show massive electron-dense accumulations. These dense bodies may also contain aggregated proteins that damage the mitochondria and the cell (fig. 1).

Another mechanism of cellular pathology could result from damage to the mtDNA. Yeast lacking Yfh1p or Ssq1p show evidence of mtDNA damage. The mechanisms by which this damage is effected remain to be determined. Increased oxidative stress resulting from excess iron might generate point mutations in the mtDNA (Babcock et al. 1997). Alternatively, mtDNA instability might result from decrease in a critical protein required for transcription or translation of the mtDNA. Hsp70 proteins are required for phage head replication (DnaK) and mtDNA replication in trypanosomes (Engman et al. 1989). In humans, various mutations in the mtDNA, frequently present in the heteroplasmic state, have been linked to neurodegenerative diseases. For example, an A to G point mutation in the mitochondrial $tRNA^{lys}$ causes myoclonic epilepsy and ragged red fiber syndrome (Chomyn 1998). Individuals with this disorder have symptoms of epilepsy and ataxia, with skeletal muscle myopathy. mtDNA damage has not yet been assessed in the affected tissues of patients with FA.

Human Diseases Revisited

The homology (and analogy) between the yeast *YFH1* and human *FRDA1* calls attention to the cellular abnormalities in FA. However, the disease affects some cells and not others, even though frataxin is ubiquitously expressed. The basis for this tissue specificity of the disease is not clear. The study of another yeast gene may shed light on this question. *ATM1* was cloned on the basis of its homology with the ATP binding cassette family of transporters. Deletion of the gene leads to mtDNA damage, iron accumulation in mitochondria, and mitochondrial iron protein deficiency (Kispal et al. 1997). Thus, the phenotypes resemble those of the *yfh1* and *ssq1* mutants. However, the human homologue of this gene recently was cloned and was found to map to Xq13, a region that has been linked to an inherited human disease called "X-linked sideroblastic anemia with ataxia" (Allikmets et al. 1998; Shimada et al. 1998). This disease is characterized by spinocerebellar ataxia, but patients also manifest a mild sideroblastic anemia. The presence of ringed sideroblasts is the hallmark of iron accumulation within mitochondria of red-cell precursors and has not been described in patients with FA.

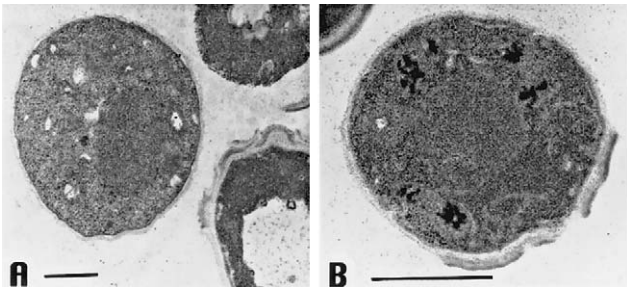


Figure 1 Electron micrographs of mutant and wild-type yeast. *A*, Wild-type yeast. *B*, The ssq1 mutant. In the mutant, the iron accumulations are visualized as electron-dense aggregates within mitochondria. Bars represent 1 μm.

FA was a clinical diagnosis prior to the cloning of the gene. The spectrum of disease must be reevaluated, now that individuals can be assessed for the presence of mutations in *FRDA1*. The question arises whether variants of FA that result from mutations in other genes will be identified. A number of ataxia variants or phenocopies of FA have been recognized. Although the majority of these have been linked to *FRDA1*, some have not. Ataxia with isolated vitamin E deficiency (AVED) is an autosomal recessive neurodegenerative disorder with a phenotype that closely resembles that of FA. Yet despite the similar symptoms, AVED is attributed to frameshift or missense mutations in the gene encoding the α-tocopherol transfer protein on chromosome 8 (Ouahchi et al. 1995). The biochemical link between vitamin E deficiency and iron is not entirely clear, although vitamin E is a potent antioxidant and might protect against the oxidative stress from iron overload.

Correct folding of proteins into their native state is critical for their proper functioning, so diseases that result from abnormalities in chaperone function are likely to exist. Ssc1p and Hsp60 are essential chaperone proteins of yeast mitochondria. These proteins are highly conserved with humans, and thus mutant alleles might be implicated in disease causation. To date, only a few reports have associated mitochondrial chaperones with disease pathology. Mitochondria from two unrelated girls, one of whom died 2 d after birth and the other at age 4.5 years, were noted to have markedly reduced levels of mitochondrial Hsp60 (Briones et al. 1997). The morphology of the mitochondria was characterized by disruptions of the inner membranes and an absence of cristae. At the biochemical level, activities of some of the tricarboxylic enzymes from cultured fibroblasts were substantially lower than activities in controls, although the Fe-S enzyme aconitase was not assayed. A Spanish family with two members who exhibit the classic symp-

toms of FA, in whom the disease does not segregate with *FRDA1,* was recently reported by Smeyers et al. (1996). The defective gene in this family might represent another complementation group for FA, and a human homologue of *SSQ1* might be considered as a candidate gene. To date, a human homologue of *SSQ1* has not been identified.

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