Understanding the genetic and molecular pathogenesis of Friedreich’s ataxia through animal and cellular models

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In 1996, a link was identified between Friedreich’s ataxia (FRDA), the most common inherited ataxia in men, and alterations in the gene encoding frataxin (FXN). Initial studies revealed that the disease is caused by a unique, most frequently biallelic, expansion of the GAA sequence in intron 1 of FXN. Since the identification of this link, there has been tremendous progress in understanding frataxin function and the mechanism of FRDA pathology, as well as in developing diagnostics and therapeutic approaches for the disease. These advances were the subject of the 4th International Friedreich’s Ataxia Conference held on 5th-7th May in the Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France. More than 200 scientists gathered from all over the world to present the results of research spanning all areas of investigation into FRDA (including clinical aspects, FRDA pathogenesis, genetics and epigenetics of the disease, development of new models of FRDA, and drug discovery). This review provides an update on the understanding of frataxin function, developments of animal and cellular models of the disease, and recent advances in trying to uncover potential molecules for therapy.

Introduction

Friedreich’s ataxia (FRDA) is the most prevalent form of hereditary ataxia in Caucasians, accounting for 75% of ataxia with onset prior to 25 years of age (Cossee et al., 1997). It is a neurodegenerative disease characterized by progressive spino-cerebellar and sensory ataxia with absence of deep tendon reflexes, dysarthria, pyramidal signs, muscular weakness and positive extensor plantar response (Harding, 1981; Pandolfo, 2009). Most of the neurological symptoms result from neurodegeneration in the dorsal root ganglia (DRG), with loss of large sensory neurons and posterior columns, followed by degeneration in the spino-cerebellar and corticospinal tracts of the spinal cord. The dentate nucleus of the cerebellum is also affected, accounting for the cerebellar phenotype (Koeppen, 2011). Primary non-neurological manifestations of the disease include hypertrophic cardiomyopathy and increased incidence of diabetes (Harding and Hewer, 1983). Although the neuropathology has been the main focus of clinical research, understanding the pathological outcomes of the cardiac and metabolic phenotypes is an emerging field. Early studies in individuals with FRDA showed that three main biochemical features characterize the pathophysiology: intracellular iron deposits (Lamarche et al., 1980), a deficit in mitochondrial iron-sulfur (Fe-S)-cluster-containing enzymes (aconitase and respiratory chain complexes I-III) (Rotig et al., 1997), and the presence of markers of oxidative damage in blood and urine samples (Emond et al., 2000; Schulz et al., 2000; Bradley et al., 2004).

The mutated gene in FRDA, which is localized on 9q21.11, encodes a small mitochondrial protein called frataxin (FXN) (Campuzano et al., 1996; Campuzano et al., 1997; Koutnikova et al., 1997). All individuals with FRDA carry an expansion of a GAA-triplet repeat in the first intron of the FXN gene. Most FRDA individuals are homozygous for this mutation, but a few patients (4%) are compound heterozygous for the GAA expansion and a different mutation (nonsense, missense, deletions, insertions), leading to loss of frataxin function (Campuzano et al., 1996; Cossee et al., 1999; Gellera et al., 2007). Normal chromosomes contain up to 40 GAA repeats, whereas disease-associated alleles contain 100-1000 GAA repeats, most commonly ~600-900. This GAA expansion leads to transcriptional silencing of FXN through a mechanism involving heterochromatinization of the locus, resulting in expression of a structurally and functionally normal frataxin but at levels that are estimated at ~5-30% of normal (reviewed in Gottesfeld, 2007; Schmucker and Puccio, 2010). As demonstrated in knockout animals, complete absence of frataxin leads to early embryonic death (Cossee et al., 2000). The other rare mutations in FXN that have been associated with FRDA lead to the production of nonfunctional or partially functional proteins (Correia et al., 2008). In most cases, compound heterozygous patients are indistinguishable from individuals who are homozygous for GAA expansions, although a few missense mutations (e.g. G130V, D122Y, D137N, etc.) lead to decreased frataxin stability and function.

Since the identification of this link, there has been tremendous progress in understanding frataxin function and the mechanism of FRDA pathology, as well as in developing diagnostics and therapeutic approaches for the disease. These advances were the subject of the 4th International Friedreich’s Ataxia Conference held on 5th-7th May in the Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France. More than 200 scientists gathered from all over the world to present the results of research spanning all areas of investigation into FRDA (including clinical aspects, FRDA pathogenesis, genetics and epigenetics of the disease, development of new models of FRDA, and drug discovery). This review provides an update on the understanding of frataxin function, developments of animal and cellular models of the disease, and recent advances in trying to uncover potential molecules for therapy.

PERSPECTIVE

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R165P, L106S) cause atypical or milder clinical presentations (Cossee et al., 1999; Gellera et al., 2007).

The genetic basis of FRDA raises challenges for modeling the disease in other species. However, in the past 15 years, since the link between FRDA and FXN was identified, many models of FRDA have been generated that have enabled advances in understanding the function of frataxin, the pathophysiology of the disease, and some of the mechanisms implicated in GAA-based silencing and instability. Owing to its high evolutionary conservation, the effect of frataxin depletion has been modeled in diverse organisms, including yeast (Babcock et al., 1997; Foury and Cazzalini, 1997), invertebrates [Caenorhabditis elegans (Vazquez-Manrique et al., 2006; Ventura et al., 2006; Zarse et al., 2007) and Drosophila (Anderson et al., 2005; Llorens et al., 2007)] and mice (Puccio et al., 2001; Miranda et al., 2002; Al-Mahdawi et al., 2006). Yeast, invertebrates and the zebrafish (Danio rerio) have been shown to be well suited to the large-scale screening of drugs (Giacomotto and Segalat, 2010). However, owing to the complexity of the clinical phenotype of individuals with FRDA and the species specificity of certain fundamental pathways, mouse or mammalian cells are probably better suited to answer pathophysiological questions. Quite unexpectedly, generating mouse models using conditional approaches as well as GAA-based mouse models (Puccio et al., 2001; Miranda et al., 2002; Al-Mahdawi et al., 2006) was more successful than the design of stable cellular models using disease-relevant cell types. Current efforts in the FRDA field aim to generate better models that genetically reproduce the partial frataxin deficiency, mainly via the introduction of GAA-expansion-containing constructs in either mouse models or cells, or by the use of induced pluripotent stem cell (iPSC)-derived cells.

This Perspective discusses the current state of research in the field of FRDA based on published data as well as on the most recent results presented during the 4th International Friedreich’s Ataxia Scientific Conference, held in May 2011 at the Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France. We begin by addressing current data and controversies regarding the role of frataxin in Fe-S cluster biogenesis, and then review the available mammalian models of the disease and advances in drug development for FRDA.

**Frataxin is a key player in Fe-S cluster biogenesis**

Frataxin is a small globular protein localized in eukaryotic mitochondria; the function of frataxin remains unclear and quite controversial. Although published data indicate that frataxin might be a multifunctional protein involved in different mitochondrial pathways, recent data combining in vitro and in vivo approaches suggest that the role of frataxin in Fe-S cluster biogenesis defines the essential function of the protein. Fe-S clusters are small inorganic cofactors that are involved in many essential cellular pathways ranging from mitochondrial respiration and metabolic processes to DNA synthesis and repair (Lill, 2009).

Early in vitro studies of bacterial, yeast or human frataxin showed a low affinity for iron (μM range), with iron interacting with a conserved acidic ridge of the mature protein (Nair et al., 2004; Cook et al., 2006). Iron binding by frataxin in vivo under physiological conditions remains to be demonstrated. Early pathophysiological studies in a conditional mouse model reproducing the heart cardiomyopathy of FRDA (MCK-Cre; Table 1) pointed to a primary involvement of frataxin in Fe-S cluster biogenesis (Puccio et al., 2001). Furthermore, phylogenetic studies predicted a role for frataxin in Fe-S cluster metabolism (Huynen et al., 2001). A role for frataxin in Fe-S cluster biogenesis was later confirmed in yeast depleted of the yeast homolog of frataxin (known as Yfh1) (Duby et al., 2002; Muhlenhoff et al., 2002).

De novo Fe-S cluster biogenesis is a highly conserved but still poorly characterized process that occurs in mitochondria in eukaryotes [see review from Lill (Lill, 2009) and commentary from Rouault in this issue of Disease Models & Mechanisms (Rouault, 2012)]. The first step of Fe-S cluster biogenesis involves the assembly of inorganic iron and sulfur into an Fe-S cluster on a scaffold protein, ISCU (Isu1 in yeast). In this process, the sulfur is provided through a persulfide intermediate by a cysteine desulfurase (comprising NFS1 and ISD11). The iron-dependent interaction of yeast frataxin with Nfs1 and Isu1 (Gerber et al., 2003), as well as in vitro reconstitution experiments showing the capacity of human frataxin to transfer iron to ISCU (Yoon and Cowan, 2003) and the capacity of CyaY (the bacterial homolog of frataxin) to provide iron for Fe-S cluster formation (Layer et al., 2006), led to the hypothesis that frataxin might be the iron donor for the assembly of the Fe-S cluster in vivo. Although controversial data regarding the direct frataxin protein partner were subsequently reported (Gerber et al., 2003; Layer et al., 2006; Shan et al., 2007; Li et al., 2009), very recent independent work using mammalian recombinant proteins reconciled the different results by showing that frataxin interacts with a preformed complex composed of Nfs1, ISCU and ISD11 (Fig. 1) (Tsai and Barondeau, 2010; Schmucker et al., 2011). Furthermore, this interaction leads to increased cysteine desulfurase activity, suggesting that frataxin modulates the capacity of Nfs1 to provide sulfur for Fe-S cluster formation (Tsai and Barondeau, 2010). In addition, although the presence of iron has been suggested to improve the activation of Nfs1 cysteine desulfurase activity by frataxin (Tsai and Barondeau, 2010), the metal is not required for the interaction between frataxin and the ISCU-NFS1-ISD11 complex (Schmucker et al., 2011). Nuclear magnetic resonance (NMR) analysis of yeast homologs of frataxin suggested that the region of interaction with Isu1 encompasses the α1-β1 region previously shown to bind iron in vitro, as well as a large section of the β-sheet of Yfh1 (Cook et al., 2010). These results are consistent with the region of interaction determined by mutagenesis experiments for mouse frataxin and the ISCU-NFS1-ISD11 complex (Schmucker et al., 2011). Furthermore, data using isothermal titration calorimetry (ITC) provided evidence that the interaction between yeast Yfh1 and Isu1 (in the absence of Nfs1-Isd11) depends on iron binding (Cook et al., 2010). Thus, the data from the yeast system suggest that the binding of iron to frataxin is part of the process leading to Fe-S cluster formation. However, the issue of how iron delivery and modulation of NFS1 activity are coordinated during Fe-S cluster formation still needs to be addressed.

Recent results obtained with the bacterial homologs bring further questions. Although CyaY, the bacterial homolog of frataxin, was shown to display a high affinity for the preformed IscS-IscU complex (as for the mammalian complex) (Prischi et al., 2010), in vitro reconstitution experiments indicated that CyaY displays an inhibitory effect on Fe-S cluster biogenesis rather than activating Fe-S cluster formation (as suggested for the mammalian proteins) (Adinolfi et al., 2010).
Moreover, in stoichiometric conditions, CyaY was found to decrease IscS enzymatic activity in the presence of IscU, thus indicating that the effect of CyaY on IscS activity is opposite to that in the mammalian system (Iannuzzi et al., 2011). The reasons for such differences and the consequences of such activation or inhibition on the capacity of the complex to form Fe-S clusters must be further investigated. New in vitro biochemical experiments as well as three-dimensional structure determination of the eukaryotic and bacterial

Table 1. Animal and cellular models of FRDA

| Model/genotype       | Notes/phenotype                                                                 | References             |
|----------------------|---------------------------------------------------------------------------------|------------------------|
| Fxn-knockout mouse   | Embryonic lethality during gastrulation (E6.5)                                  | Cossee et al., 2000    |
| **Conditional mouse models of Fxn deletion** |                                                                      |                        |
| MCK-Cre              | Muscle creatine kinase promoter. Fxn deletion in heart and skeletal muscle. Reduced lifespan (76±10 days) and hypertrophic cardiomyopathy but no skeletal muscle phenotype. Early Fe-S cluster deficit and late mitochondrial iron accumulation. No sign of oxidative stress | Puccio et al., 2001    |
| NSE-Cre              | Neuron-specific enolase promoter. Fxn deletion in nervous system, heart and liver. Reduced lifespan (29±9 days). Severe neuronal and cardiac phenotype | Puccio et al., 2001    |
| PrP-CreER            | Tamoxifen-inducible Cre, prion promoter. Fxn deletion in DRG and cerebellum. Progressive spinocerebellar and sensory ataxia. Neurodegeneration of sensory neurons in DRG and granular layer in cerebellum. Abnormal autophagy in DRG | Simon et al., 2004     |
| Ins2-Cre             | Insulin promoter. Fxn deletion in pancreatic β-cells; diabetes mellitus         | Ristow et al., 2003    |
| ALB-Cre              | Albumin promoter. Fxn deletion in hepatocytes. Tumor formation or liver regeneration? | Thierbach et al., 2005 |
| **Mouse models with GAA expansions in Fxn** |                                                                      |                        |
| KIKI                 | Double knock-in with 230 GAA repeats. No overt phenotype. Transcriptional deregulation involving the PPAR pathway. Markers of heterochromatin on the GAA tract | Miranda et al., 2002   |
| KIKO                 | Simple knock-in crossed with knockout mouse. 26-32% residual frataxin expression. No overt phenotype. Transcriptional deregulation involving the PPAR pathway | Miranda et al., 2002   |
| YGBR                 | YAC containing the full human Fxn locus with a GAA expansion and deleted for endogenous murine frataxin. Progressive ataxia with affected DRG. No cardiopathy but mitochondrial iron accumulation and lipid peroxidation. Markers of heterochromatin on the GAA tract. Tissue-dependent GAA instability | Al-Mahdawi et al., 2006 |
| **Non-mammalian animal models** |                                                                      |                        |
| Drosophila melanogaster | Generated using RNAi to knock down Fxn expression. Reduced lifespan, Fe-S cluster and heme deficit, sensitivity to oxidative stress, climbing defect. Catalase or peroxiredoxin overexpression does not rescue the phenotype in larvae, but partially rescues the phenotype in the adult fly | Anderson et al., 2005  |
| C. elegans           | Generated using RNAi to knock down FXN expression. Controversial results depending on experimental procedures | Ventura et al., 2006   |
| Zebrafish            | Generated using antisense strategies to knock down fxn expression. Affects development of several tissues (e.g. ear, spinal motor neurons) depending on frataxin depletion level | Constantin Yanicoustas* |
| **Cellular models**  |                                                                      |                        |
| Escherichia coli     | Deletion of CyaY does not lead to an overt phenotype                          | Li et al., 1999        |
| Saccharomyces cerevisiae | Deletion of Yfh1 leads to Fe-S cluster deficit, mitochondrial iron accumulation and increased sensitivity to oxidative stress | Foury and Cazzalini, 1997 |
| Patient-derived fibroblasts or lymphoblasts | ~20-30% residual frataxin expression. No observed phenotype in normal culture conditions. High variability | Rotig et al., 1997 |
| RNAi-based models    | Many different cell lines. Variable residual frataxin expression. Fe-S cluster deficit, cell proliferation defect, cell death | Santos et al., 2001    |
| Humanised murine fibroblasts | Murine fibroblasts deleted for endogenous frataxin and expressing human frataxin with missense mutation (G130V, I154F or N146K). Fe-S cluster deficit, mitochondrial iron accumulation and/or deposits, sensitivity to oxidative stress | Calmels et al., 2009    |
| iPSC-derived cells   | Patient-derived iPSCs show decreased FXN expression and markers of heterochromatin at the GAA expansion. GAA expansion instability was observed in iPSCs. Protocols to obtain specific neuronal populations and cardiomyocytes are currently being developed | Ku et al., 2010        |

*Personal communication, as presented at the 4th International Friedreich’s Ataxia Conference (http://www.curefa.org/conference.html).
complexes using X-ray crystallography will bring additional and complementary valuable information that will enable a better understanding of the role of frataxin in Fe-S cluster biogenesis. Recently, a point mutation in the Isu1 gene (M107I) was found to partially rescue the ΔYfh1 yeast model, pointing again to the role of frataxin in the early steps of Fe-S cluster biogenesis (Yoon et al., 2012). Further molecular and biochemical studies aimed at understanding how this point mutation affects the activity of the Isu1-Nfs1-Isd11 complex in the absence of frataxin should bring new insights concerning the role of frataxin.

The finding that frataxin affects Nfs1 activity opened up the possibility to assess the functional consequences of missense mutations identified in individuals with FRDA. Four different classes of frataxin mutations were identified on the basis of their biochemical properties (Bridwell-Rabb et al., 2011; Tsai et al., 2011): (1) variants that affect both frataxin binding to the ISCU-NFS1-ISD11 complex and its cysteine desulfurase activity (Q153A, W155R); (2) variants that display weak binding but have only a mild reduction in their capacity to activate Nfs1 (R165C, N146K, W155A); (3) variants for which binding is not markedly affected but that show a substantial defect in their activation of cysteine desulfurase (Q148R); and (4) variants that display modest defects in both binding and activation (W155F, N146A, Q148G, I154F). These results show that missense mutations affecting the function of frataxin through different biochemical mechanisms can ultimately lead to a similar phenotype. In the future, it would be interesting to assess how variants that lead to atypical disease (G130V, D122Y, R165P, L106S) (Cossee et al., 1999; Gellera et al., 2007) affect the process of Fe-S cluster formation.

In addition to its clear role in Fe-S cluster biogenesis, data suggest that frataxin might be a multifunctional protein involved in providing iron through direct protein-protein interaction to various mitochondrial proteins (Fig. 1), including mitochondrial aconitase (Bulteau et al., 2004), ferrochelatase (He et al., 2004; Yoon and Cowan, 2004) and succinate dehydrogenase (Gonzalez-Cabo et al., 2004), providing iron through direct protein-protein interaction to various mitochondrial proteins (Fig. 1), including mitochondrial aconitase (Bulteau et al., 2004), ferrochelatase (He et al., 2004; Yoon and Cowan, 2004) and succinate dehydrogenase (Gonzalez-Cabo et al., 2004), but that show a substantial defect in their activation of cysteine desulfurase activity (Q153A, W155R); (2) variants that display weak binding but have only a mild reduction in their capacity to activate Nfs1 (R165C, N146K, W155A); (3) variants for which binding is not markedly affected but that show a substantial defect in their activation of cysteine desulfurase (Q148R); and (4) variants that display modest defects in both binding and activation (W155F, N146A, Q148G, I154F). These results show that missense mutations affecting the function of frataxin through different biochemical mechanisms can ultimately lead to a similar phenotype. In the future, it would be interesting to assess how variants that lead to atypical disease (G130V, D122Y, R165P, L106S) (Cossee et al., 1999; Gellera et al., 2007) affect the process of Fe-S cluster formation.

Frataxin has been shown to form oligomers in the presence of iron in vitro (Adamec et al., 2000) (Fig. 1). The formation and function of oligomeric frataxin in vivo is, however, still a matter of debate. In vitro reconstitution experiments showed that the oligomeric forms of frataxin are important in providing iron for Fe-S cluster biogenesis. More recently, the oligomeric forms of frataxin protein partners, as well as the role of the oligomeric form of frataxin in vivo, were questioned (see text). Indeed, a tight and stable iron-independent complex between monomeric frataxin and the ISCU-NFS1-ISD11 complex was isolated (represented by solid arrows), and the ability of frataxin to form this complex was shown to correlate with the essential frataxin function in vivo (Tsai and Barondeau, 2010; Schmucker et al., 2011). Further studies showed that, on frataxin binding, the cysteine desulfurase activity of the ISCU-NFS1-ISD11 complex is increased (red arrow), and Fe-S cluster biogenesis on ISC is enhanced, suggesting that frataxin is a key modulator of de novo Fe-S cluster formation in vivo. PPIX, protoporphyrin IX.

Fig. 1. Frataxin is a mitochondrial protein with a key role in Fe-S cluster biogenesis. Over the past 10 years, frataxin has been proposed to be a multifunctional protein involved in providing iron to various mitochondrial proteins (represented by dashed arrows), including succinate dehydrogenase (SDH), mitochondrial aconitase (mACO) and ferrochelatase (FCH), as well as for Fe-S cluster biogenesis, which involves the cysteine desulfurase NFS1-ISD11 and the scaffold protein ISCU. SDH, mACO and FCH are Fe-S-containing proteins in mammals. Frataxin has also been proposed to form oligomeric structures that can store iron. In vitro, both monomeric and oligomeric forms of frataxin can provide iron for Fe-S cluster biogenesis. More recently, the existence of multiple frataxin protein partners, as well as the role of the oligomeric form of frataxin in vivo, were questioned (see text). Indeed, a tight and stable iron-independent complex between monomeric frataxin and the ISCU-NFS1-ISD11 complex was isolated (represented by solid arrows), and the ability of frataxin to form this complex was shown to correlate with the essential frataxin function in vivo (Tsai and Barondeau, 2010; Schmucker et al., 2011). Further studies showed that, on frataxin binding, the cysteine desulfurase activity of the ISCU-NFS1-ISD11 complex is increased (red arrow), and Fe-S cluster biogenesis on ISC is enhanced, suggesting that frataxin is a key modulator of de novo Fe-S cluster formation in vivo. PPIX, protoporphyrin IX.
Bypassing the formation of the intermediate human frataxin in vivo using ‘humanized’ murine fibroblasts deleted for endogenous frataxin does not prevent the rescue of cellular viability, thus indicating that the formation of oligomers is not a requisite for frataxin to be functional (Schmucker et al., 2011). We cannot exclude, however, that an oligomeric form of frataxin is of particular importance in specific cellular conditions that have yet to be determined.

Understanding the function of frataxin is crucial to unravelling the cellular consequences of its depletion in vivo. A key function of frataxin in Fe-S cluster biogenesis has now been clearly pointed out, but how its role in this essential cellular pathway correlates with the pathophysiology of FRDA needs to be further investigated through the study of well-characterized animal and cellular models of the disease. During the last 10 years, different approaches in cells, mice and non-mammalian organisms have been pursued to obtain such models.

Investigating the pathophysiology of FRDA using mouse models
Conditional knockout models
The complete knockout of \( Fxn \) in mice is lethal (Cossee et al., 2000) (Table 1). Similarly, the complete deletion of \( Fxn \) in proliferating cells is not viable (Calmels et al., 2009). The first viable mouse models of FRDA were generated using conditional approaches using the \( \text{Cre-loxP} \) recombination system to perform deletion of \( Fxn \) exon 4 from a conditional floxed allele (\( Fxn^{\text{loxP}} \)) using tissue-specific, or tissue-specific and inducible, \( \text{Cre} \) mouse lines. Cardiac-specific (\( \text{MCK-Cre} \)) and neuronal (\( \text{NSE-Cre}, \text{Prp-Cre}^{\text{ERT}} \)) models of FRDA were obtained (Puccio et al., 2001; Simon et al., 2004) (Table 1). Together, these models reproduce most of the characteristic features of the disease, including hypertrophic cardiomyopathy, progressive spinocerebellar and sensory ataxia. Time-dependent molecular and functional dissection of the \( \text{MCK-Cre} \) mouse model showed that frataxin deficiency primarily affects Fe-S cluster-containing enzymes (Puccio et al., 2001). The Fe-S cluster deficit is observed before the first evidence of cardiac dysfunction, and before the characteristic mitochondrial iron accumulation. Echocardiography demonstrated that \( \text{MCK-Cre} \) mice develop a progressive left ventricular hypertrophy that rapidly associates with geometric remodeling (dilatation) leading to cardiac failure, consistent with the natural history of the human disease (Seznec et al., 2004; Regner et al., 2011). Interestingly, no evidence of oxidative damage was observed in this model, suggesting that the formation of reactive oxygen species (ROS) is not essential in the early onset of the disease (Seznec et al., 2005).

The \( \text{NSE-Cre} \) model develops a movement disorder characterized by gait abnormalities and loss of proprioception, as well as cardiac disease (Puccio et al., 2001). However, the severity of this model and the non-specific neurological lesions (spongiform cortical lesions) compromise the use of this model for neuropathophysiological studies.

The tamoxifen-inducible \( \text{Prp-Cre}^{\text{ERT}} \) neuronal model showed that the spinocerebellar and sensory ataxia developed by the mice result from progressive degeneration of the posterior and Clarke’s columns of the spinal cord, as well as degeneration of large sensory neurons in DRG (Simon et al., 2004). A time course experiment in these mice suggested that lesions observed in DRG neurons are a primary event, whereas neuronal loss in the Clarke’s column and the posterior column are secondary. These features are reminiscent of the changes observed in individuals with FRDA (Koeppen, 2011).

In addition, although there was no evidence of apoptosis, an abnormal autophagic process (leading to the formation of lipofuscin and of large vacuoles within large sensory neurons) was observed in the \( \text{Prp-Cre}^{\text{ERT}} \) mice (Simon et al., 2004), suggesting that impaired autophagy is a key process in the neurodegeneration.

Conditional models lacking \( Fxn \) primarily in the pancreas (\( \text{Ins2-Cre} \); Table 1) were generated to model the pathophysiology of diabetes mellitus associated with FRDA (Ristow et al., 2003) (Table 1). These mice showed a slowly progressive reduction in the number of pancreatic islets, resulting in an impaired insulin response to glucose and subsequent diabetes. Frataxin-deficient \( \beta \)-cells exhibit increased apoptosis and a reduced proliferation rate (Ristow et al., 2003). However, whether the described phenotype observed in \( \text{Ins2-Cre} \) mice reproduces the pathological mechanism that occurs in individuals with FRDA needs to be further investigated through complementary studies in the mouse model and in patients.

To understand the function of frataxin in different tissues, and the tissue specificity of the FRDA phenotype, mouse lines with deletion of \( Fxn \) in non-diseased tissues have been generated. The observation of tumor-like lobules in a liver-specific deletion of \( Fxn \) (\( \text{ALB-Cre} \); Table 1) suggested that frataxin might be a tumor suppressor (Thierbach et al., 2005; Thierbach et al., 2010; Thierbach et al., 2012). However, revisiting the status of frataxin deletion and the phenotype associated with the \( \text{ALB-Cre} \) mice provides a different interpretation. In fact, the formation of lobules in the liver, which is associated with increased lifespan, seemed to be the consequence of liver regeneration from ALB-naïve precursors expressing frataxin (Diaz et al., 2008; Iverson et al., 2011) (A.M. and H.P., unpublished data). Thus, the suggested tumor suppressor role of frataxin should be considered with care. Consistent with this observation, data from a large cohort of individuals with FRDA did not demonstrate an increased tumor incidence in these individuals (David R. Lynch, personal communication, as presented at the 4th International Friedreich’s Ataxia Conference (http://www.curefa.org/conference.html]).

GAA-based mouse models
Although conditional knockout mice are powerful tools to reproduce the disease pathophysiology in heart and the nervous system, and to test some therapeutic approaches, they do not perfectly mimic the human disease. In individuals with FRDA, the presence of a GAA expansion on at least one \( FXN \) allele leads to low levels of structurally normal frataxin (Campuzano et al., 1996). The progressive disease thus results from the presence of a residual amount of frataxin throughout life, rather than a sudden and complete frataxin deficit (as triggered by the conditional knockout mouse approach). Furthermore, the genetics of GAA expansions might contribute to disease development, possibly by having a role in tissue specificity owing to the intrinsic somatic instability of long GAA tracts (see Box 1). In addition, GAA-based mouse models are needed to unravel the molecular and cellular mechanisms associated with GAA-mediated silencing of the \( FXN \) gene in vivo (see Box 2) as well as for the therapeutic evaluation of drug candidates that might target this process.

GAA tracts (see Box 1). In addition, GAA-based mouse models are needed to unravel the molecular and cellular mechanisms associated with GAA-mediated silencing of the \( FXN \) gene in vivo (see Box 2) as well as for the therapeutic evaluation of drug candidates that might target this process.
GAA-based mouse models were obtained using either a knock-in approach based on homologous recombination or a human genomic yeast artificial chromosome (YAC) transgenic approach. Homozygous 230 GAA-repeat-expansion knock-in mice (KIKI) show a 25% decrease in frataxin expression, whereas the compound heterozygous knock-in–knockout mice (KIKO) express 25–35% of wild-type frataxin levels (Miranda et al., 2002) (Table 1). No overt phenotype was observed in the KIKO mice, suggesting that the transcriptional decrease does not reach the critical frataxin threshold required to induce pathology. However, transcriptome analysis of both KIKI and KIKO mice revealed significant transcriptional modifications in these mice compared with control mice (Coppola et al., 2009). In particular, the PPARγ pathway was affected (i.e. PGC1α was downregulated) in liver and muscle samples. However, controversial data regarding dysregulated PGC1α expression in patient fibroblasts (Marmolino et al., 2010; Garcia-Gimenez et al., 2011) have recently been reported, showing that further studies are needed to understand the link between frataxin deficiency and metabolic control. Although this link still needs to be defined, the cellular mechanisms involved might be of particular interest when trying to understand the development of diabetes mellitus in individuals with FRDA.

Two lines of human genomic YAC FRDA transgenic mice (YG22 and YG8) that contain unstable GAA-repeat expansions (GAA_{190} and GAA_{190–90}, respectively), within the appropriate genomic context, rescue the embryonic lethality of the knockout by expressing only human frataxin (Al-Mahdawi et al., 2006). The YG8 rescued mice, referred to as YG8R (Table 1), exhibit reduced expression of human frataxin protein and display mildly impaired motor coordination ability, with evidence of large neurodegeneration vacuoles in the DRG and decreased aconitase activity in the cerebellum. These features are reminiscent of those observed in NSE-Cre and early-stage Prp-CreER² mice (Puccio et al., 2001; Simon et al., 2004). No severe heart dysfunction was observed in YG8R mice. However, decreased aconitase activity, mitochondrial iron accumulation and signs of lipid peroxidation suggestive of ROS formation were found in heart tissue (Al-Mahdawi et al., 2006). Microarray analysis carried out on DRG from YG8R mice showed that the expression of thiol-related proteins (e.g. thioredoxin reductase, peroxiredoxins) was impaired in affected mice compared with controls [Robert Schoenfeld, personal communication, as presented at the 4th International Friedreich's Ataxia Conference (http://www.curefa.org/conference.html)]. Although the role of this pathway in the pathophysiology still needs to be clearly defined, the hypothesis was raised that excessive thiol oxidation is a key feature leading to demyelination and neurodegeneration in YG8R mice.

Generating adequate GAA-based models that lead to the development of an FRDA phenotype in a mouse is not an easy task. The main difficulties lie in the length of the GAA expansion needed to induce pathogenesis and the locus of genomic integration, as well as the intrinsic GAA instability that can result in contraction or elongation of the expansion. Both KIKI and YG8R mice are powerful tools to study the mechanisms involved in GAA-mediated silencing and GAA somatic instability (YG8R) (see Boxes 1 and 2). To obtain new mouse models with a more severe phenotype, current efforts are being made to increase the size of the GAA expansion within the human FXN transgene. Furthermore, bacterial artificial chromosomes (BACs) that can accommodate the full FXN locus with larger GAA expansions are currently being generated. These constructs will certainly be valuable tools for developing new mouse models.

Further dissecting the molecular consequences of frataxin depletion in models

Many aspects of the molecular pathophysiology of FRDA remain to be addressed using the different mouse models generated. For instance, in both Prp-CreER² and YG8R mice, additional molecular and cellular investigations are needed to fully understand the process(es) leading to neurodegeneration. Although autophagy has been identified as being involved in the neurodegeneration of large sensory neurons of DRG (Simon et al., 2004; Al-Mahdawi et al., 2006), the molecular and cellular mechanisms leading to this phenomenon are not known.

Although the role of oxidative stress in pathology remains an open controversial question, a clear iron dysregulation (mainly illustrated by mitochondrial iron accumulation) is observed in FRDA (Lamarche et al., 1993; Michael et al., 2006; Koeppen et al., 2007). Understanding the molecular mechanisms involved in such dysregulation, as well as determining the role of this iron accumulation in pathophysiology, is important. Iron chelators are indeed thought to be potential therapeutic agents for FRDA (Richardson, 2003), and deferiprone is currently being tested in clinical trials. Therefore, understanding exactly how frataxin depletion leads to iron dysregulation might help in evaluating the relevance of such therapeutic approaches. Data obtained from the

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**Box 1. Mechanism of somatic instability of GAA expansions**

Work with patient samples and GAA-based models has provided evidence of age-dependent, tissue-specific somatic instability and intergeneration instability of the expanded GAA repeats in FRDA (Montermini et al., 1997; Sharma et al., 2002; Pollard et al., 2004; De Biase et al., 2006). An increase in the repeat size might be an important contributor to the pathology of the disease because the size of the repeat expansion correlates with the extent of FXN expression impairment. Hence, preventing expansion of the GAA repeats represents a potential therapeutic goal for FRDA. In FRDA iPSCs, a high occurrence of both GAA repeat expansions and contractions were reported (Ku et al., 2010; Liu et al., 2011). GAA instability was reported to be dependent on increased activity of the mismatch repair system (especially Msh2) during the reprogramming and subsequent culturing of FRDA iPSCs (Ku et al., 2010). Interestingly, mismatch repair proteins were also found, using chromatin immunoprecipitation (ChIP), in the vicinity of the expanded GAs in intron 1 of FXN. Accordingly, the importance of mismatch repair proteins Msh2 and Msh3, but not Msh6, was underlined in inducing repeat instability in model cell lines containing expanded GAs [Edward Grabczyczk, personal communication as presented at the 4th International Friedreich’s Ataxia Conference (http://www.curefa.org/conference.html)]. Mismatch repair protein expression also affects intergenerational instability of the GAA repeats in vivo. Indeed, crossing Msh2- or Msh3-knockout mice with YG8R mice (see main text) led to a significant increase in the occurrence of repeat contractions in the offspring compared with YG8R mice, whereas crossing YG8R mice to Msh6 knockouts had the opposite effect, increasing the incidence of expansions [Yahid Ezzatiadzeh, personal communication as presented at the 4th International Friedreich’s Ataxia Conference (http://www.curefa.org/conference.html)]. Similar to prior studies conducted with other trinucleotide repeat sequences (Lin et al., 2009), transcription through the GAA repeat seems to stimulate instability (Ditch et al., 2009). However, contrary to the studies on CTG repeats, the nucleotide excision repair (NER) pathway did not affect GAA repeat instability.
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Box 2. GAA-expansion-mediated silencing of FXN

Expanded GAA tracts confer variegation of expression of a linked transgene in murine cells (Saveliev et al., 2003). This silencing is enhanced by heterochromatin protein 1 (HP1) and is associated with increased nucleosome occupancy in the GAA repeat region. Expanded pathological GAA tracts in cells from individuals with FRDA show increased levels of histone H3 methylation (H3K9me2 and H3K9me3) – which is associated with silenced heterochromatin – and decreased abundance of acetylation at histone H3 and H4 (e.g. H3K14ac, H4K5ac, H4K12ac), which are epigenetic markers for active transcription (Herman et al., 2006). These chromatin changes were detected downstream and upstream of the GAA expansion. The aberrant chromatin status was confirmed in various models, including patient lymphoblast and fibroblast cell lines, iPSCs derived from FRDA patient cells, iPSC-derived neurons, patient autopsies tissues, GAA-based mouse models and genome-integrated transgenes containing long GAA repeats (Gottesfeld, 2007; Greene et al., 2007; Al-Mahdawi et al., 2008; Soragni et al., 2008; Ku et al., 2010; Kim et al., 2011; Sandi et al., 2011). Changes in histone modifications are associated with increased CpG methylation in intron 1 of FXN in patient cells (Castaldo et al., 2008). Although the involvement of epigenetic changes in FRDA etiology is undisputed, the exact extent of the epigenetic changes remains unclear. Some results indicate that the expansion predominantly affects transcriptional elongation (Herman et al., 2006; Kim et al., 2011; Punja and Buhler, 2011), which could be attributed to a physical barrier generated by a long polypurine-polypyrimidine sequence and/or by the formation of non-canonical DNA structures. Other data suggest spreading of the heterochromatinization towards the promoter region, consequently affecting the initiation of transcription (De Biase et al., 2009; Kumari et al., 2011). Differences between the results of chromatin immunoprecipitation (ChIP) analyses might arise from great diversity between samples (variation of FXN expression from 5% to 30%; size of GAA expansion), as well as inherent variations associated with the ChIP technique. Furthermore, an unusual stalling of RNA polymerase II (RNAPII) in exon 1 of FXN was recently identified (Changir Yandim, personal communication as presented at the 4th International Friedreich’s Ataxia Conference (http://www.curefa.org/conference.html)). Stalled RNAPII was phosphorylated at serine 2, which is a characteristic of the actively elongating enzyme. Although the exact role of the pause site is not clear, stalling of RNAPII was associated with recruitment of the proteasome and the insulator protein CTCF. Analyses of a large patient and control cohort supported by high-throughput second-generation sequencing would help to clarify the extent of epigenetic changes in FRDA cells. These analyses should include RNAPII and CTCF occupancy studies. In addition, the mediator that links GAA expansion with chromatin and DNA methylation changes and triggers the cascade of epigenetic changes has not yet been identified. New evidence suggests the involvement of RNA-mediated transcriptional gene silencing (TGS) initiated by FXN mRNA and its antisense counterpart FAST-1 (Yogesh Chutake, personal communication as presented at the 4th International Friedreich’s Ataxia Conference (http://www.curefa.org/conference.html)). Future experiments will determine the potential role of dsRNA-mediated TGS in silencing FXN expression.

Reproducing the genetic defect of FRDA in cellular models

Appropriate cell culture models of FRDA are extremely important to uncover the molecular mechanisms of the disease as well as for testing novel therapeutic approaches. Many cellular studies of FRDA pathogenesis were, and are still, conducted using patient-specific immortalized lymphoblasts, primary fibroblasts or peripheral lymphocytes. Although these patient-derived cells recapitulate some molecular features of the disease, including the epigenetic silencing of FXN expression (Box 2), they do not develop spontaneous phenotypic characteristics of FRDA (Rotig et al., 1997; Sturm et al., 2005). In an attempt to generate more relevant disease models (i.e. low levels of frataxin expression), different cell lines (fibroblasts, neuronal cell lines, Schwann cells, etc.) have been manipulated to reduce frataxin expression using siRNA- or shRNA-mediated silencing (Santos et al., 2001; Stehling et al., 2004; Napoli et al., 2007; Zanella et al., 2008; Lu et al., 2009). Different, inconsistent phenotypes have been observed in these various cellular models, probably due to the variable knockdown efficiencies (ranging from almost no detectable frataxin expression to about 40% of the residual amount of the protein), pointing to the need for stable and disease-relevant cellular models.

Two strategies have been recently developed for new cellular models of FRDA. The first is based on the exclusive expression in murine fibroblasts of a transgenic human frataxin containing a missense mutation that was identified in compound heterozygous individuals. The second strategy takes advantage of the recent advances in protocols that generate iPSCs from patient fibroblasts.

‘Humanized’ murine fibroblasts

The absence of frataxin in a murine fibroblast cell line, through deletion of the Fxnh−/− conditional allele using transient expression...
of Cre recombinase, leads to cell death (Calmels et al., 2009). This lethal phenotype can be rescued by stable transfection with a transgene expressing human frataxin or human frataxin bearing various point mutations (G130V, I154F or N146K) prior to the deletion of endogenous Fxn (Calmels et al., 2009; Schmucker et al., 2011). Whereas the expression of wild-type human frataxin led to fully functional cells in the absence of endogenous frataxin, cells expressing mutant forms of human frataxin spontaneously displayed characteristics of FRDA, including Fe-S cluster deficit, mitochondrial iron accumulation and deposits, and sensitivity to oxidative stress (Calmels et al., 2009; Schmucker et al., 2011). The severity of the cellular phenotype correlated with the severity of the mutation. Indeed, the phenotype of the cells expressing FXN<sup>G130V</sup>, a mutation that leads to a late-onset form of FRDA, was less severe than that of cells expressing FXN<sup>N154F</sup> (which is associated with a classical form of the disease) (Calmels et al., 2009). A similar approach is currently underway to generate neuronal models using neurospheres derived from conditional mice. Such approaches will help to identify and compare the pathophysiological differences between complete and partial frataxin deficit in the cells that are affected in patients. Furthermore, it will help to evaluate the functional impact of the different missense mutations identified in individuals with FRDA.

This approach could also be used to generate new mouse models. Work with fibroblasts shows, however, that these point mutations can strongly affect cellular function (Calmels et al., 2009; Schmucker et al., 2011), and therefore the mutation used to generate a mouse model with a knock-in, YAC or BAC strategy has to be chosen with caution. Alternatively, viral vectors could be used in the Fxn-knockout background to test the effect of the different mutations on the function of a specific tissue.

### Development of iPSC-derived cells

Recently, the ability to derive iPSCs from terminally differentiated human cells was a groundbreaking discovery, with alluring potential for cell-specific disease models, regenerative medicine, modeling physiological developmental processes and drug discovery (Wu and Hochedlinger, 2011). The reprogramming of somatic cells into iPSCs allows for the generation of patient-specific pluripotent cells in a manner that is entirely independent of the availability of embryonic stem cells, thus allowing the generation of multiple cell lines from a large patient pool. The FRDA-iPSC lines will have a tremendous advantage over conventional cellular models by allowing the generation of patient-specific and disease-relevant cell lineages, including neurons and cardiomyocytes.

Several iPSC lines derived from individuals with FRDA have already been generated, using different combinations of reprogramming transcription factors (Oct4, Sox2, KLF4 and Myc; or Oct4, Sox2, Nanog and Lin28) (Ku et al., 2010; Liu et al., 2011) (M.N. and H.P., unpublished data). The initial experimental data showed that these FRDA iPSCs recapitulate some of the molecular genetic aspects of FRDA, including mismatch-repair-dependent repeat expansion (see Box 1), epigenetic silencing of the FXX locus (see Box 2) and low levels of frataxin expression. The major focus of FRDA iPSC differentiation research is currently focused on generating appropriate disease-relevant cell types. For example, sensory neurons of the DRG are crucially affected in individuals with FRDA. Neural crest progenitors derived from FRDA iPSCs were successfully differentiated towards peripheral neurons with reduced levels of frataxin (Liu et al., 2011). Because cardiomyopathy is also a main feature of FRDA, more efficient protocols to obtain cells of the cardiac lineage are currently being developed. Although cardiomyocytes derived from FRDA iPSCs with low levels of frataxin were obtained (Liu et al., 2011) [Marie Wattenhofer-Donzé, personal communication as presented at the 4th International Friedreich’s Ataxia Conference (http://www.curefa.org/conference.html)], the process is rather inefficient, suggesting that modifications of current protocols and/or the application of different reprogramming strategies that could improve the differentiation potential of the iPSCs are necessary.

Future experiments will show to what extent iPSC-derived cells recapitulate pathological features observed in patient material and animal models. iPSC-based technology will also enable investigation of areas for which currently available FRDA models are not appropriate. For example, the majority of individuals with FRDA suffer from glucose intolerance, and a fraction present with diabetes (Pandolfo, 2009), but the molecular mechanisms leading to metabolic imbalance are unknown. Protocols for differentiating embryonic cells and iPSCs into pancreatic islets have recently been extensively tested (Noguchi, 2010), so FRDA iPSC-derived islet models are likely to be created soon.

Another exciting avenue of iPSC research is the possibility to generate autologous cells for regenerative therapy (Wu and Hochedlinger, 2011). In the case of FRDA, regenerative therapy could be used to correct the cardiac phenotype. Initial attempts to form fully vascularized human cardiac constructs from iPSCs that would be suitable for transplantation demonstrate the great potential for iPSC-based research for future cell-replacement therapy [Greg Dusting, personal communication as presented at the 4th International Friedreich’s Ataxia Conference (http://www.curefa.org/conference.html)]. However, in parallel with efficient differentiation and tissue development technologies, strategies to genetically correct GAA expansions in FRDA cells need to be developed.

### High-throughput drug screening for FRDA

At the present time, more than ten different therapeutic approaches aimed towards the treatment of FRDA are either entering clinical trials or are already in various stages of testing (details regarding the current status of the Friedreich’s Ataxia Treatment Pipeline can be found at http://www.curefa.org/pipeline.html). These therapeutic strategies range from iron chelators (deferiprone) and agents used to improve mitochondrial functions (e.g. idebenone or EPI-A0001), to activators of FXXN expression (e.g. HDAC inhibitors) and approaches designed to increase intracellular levels of frataxin (e.g. cEPO or TAT-frataxin) (reviewed in Tsou et al., 2009; Schmucker and Puccio, 2010).

To search for new FRDA therapeutics, high-throughput drug screening (HTS) approaches have been developed by at least ten research groups (Table 2). A lead compound might target one of several pathological aspects of FRDA, such as the transcriptional silencing of FXXN, dysregulated iron metabolism or mitochondrial defects. Regardless of the target, the robustness of the HTS assay is fundamental for success.

Because individuals with FRDA carry an intronic GAA expansion on at least one allele, leaving the entire frataxin coding sequence...
intact, the most obvious drug target is the transcriptional silencing of \( FXN \). Moreover, as is evident from asymptomatic expansion carriers and the lack of phenotype in KIKO mice (Miranda et al., 2002), a slight increase in \( FXN \) expression is likely to be therapeutic. In this context, cell-based approaches involving engineered reporter genes (luciferase or EGFP) that contain artificial intronic sequences or are conjugated to \( FXN \) have been developed for four different HTS approaches aiming to uncover novel compounds capable of frataxin upregulation (Table 2). In line with the molecular features of FRDA, the reporters harboring expanded GAA repeats demonstrated reduced expression of the reporter gene compared with constructs harboring short GAA repeats (Grant et al., 2006; Soragni et al., 2008). In some reporter assays, epigenetic changes in the vicinity of the long GAA repeats, similar to those found in the \( FXN \) gene, were detected (see Box 1). An EGFP reporter linked to the entire human \( FXN \) locus not containing pathogenic GAA expansions was also generated to discover compounds affecting expression of the \( FXN \) gene (Table 2) (Sarsero et al., 2005).

Finding compounds that alleviate frataxin insufficiency in FRDA cells by stimulating transcription or increasing protein level are not the only objectives of HTS. For example, one of the first HTS approaches for potential FRDA therapeutics was conducted in a yeast strain conditionally deficient in frataxin (Table 2) [Robert Wilson, personal communication as presented at the 4th International Friedreich’s Ataxia Conference (http://www.curefa.org/conference.html)]. Rescue of the mitochondrial functions of the yeast mutant by a large panel of compounds was monitored using a high-throughput format using the tetrazolium dye WST-1. The identified leads are now being evaluated in secondary assays using the ‘humanized’ murine fibroblast model (Calmels et al., 2009). In a similar study, a HTS approach based on the increased sensitivity to diamide (thiol oxidizing compound) of FRDA cells is being developed to uncover small molecules capable of increasing the viability of affected cells (Table 2) [Sunil Sahdeo, personal communication as presented at the 4th International Friedreich’s Ataxia Conference (http://www.curefa.org/conference.html)]. Interestingly, a novel computational-based screen was developed to identify lead compounds targeting the ubiquitin-mediated proteasomal degradation of frataxin (Rufini et al., 2011). The lead compounds showed promising results in FRDA lymphocytes.

The majority of screening efforts are currently in the initial phase of assay validation, or are gathering initial results from the HTS and validating primary hits. Thus far, no screens based on the iPSC-derived, disease-relevant cells have been developed. In the future, these approaches will be particularly important for the development of drugs to target the tissue-specific aspects of FRDA pathology.

**Conclusions**

A new era in the field of FRDA research and therapeutics is beginning. Although the generation of suitable animal and cellular models for FRDA research has been challenging – in particular because none of the models completely satisfy all aspects of the disease – studies in models have made an enormous contribution to the field and have provided the basis for many potential treatments. The availability in the near future of additional models that more closely reflect the genetic basis of the disease, such as iPSC-derived, disease-relevant cells or mice carrying longer GAA expansions, will enable refinements in our understanding of the molecular basis of the disease. Animal and cellular models will continue to be useful in discovering novel therapeutic approaches (both pharmacological and gene replacement) for treating the disease in humans.

Although trial design has been difficult owing to the slowly progressive nature of the disease, clinical trials to test drugs that aim to improve the neurological or cardiac impairment have now commenced (http://www.curefa.org/pipeline.html). Despite the lack of positive results of trials to date, FRDA remains an attractive disease target for drug development owing to the significant preclinical advances and the increasing organization of patient
groups, both at the national and international level, promoting collaboration among investigators and the development of necessary clinical trial infrastructures. There is a strong interest in identifying sensitive biomarkers that will enable earlier initiation of treatment. Together with improvements in trial design that take into account the small, diverse patient population and the variable drug-responder rate, the field of FRDA research will undoubtedly see further success in the years to come.

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COMPETING INTERESTS
The authors declare that they have no competing or financial interests.

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