Frataxin inactivation leads to steroid deficiency in flies and human ovarian cells

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Abstract

Friedreich’s ataxia (FA), the most common inherited autosomal-recessive ataxia in Caucasians, is characterized by progressive degeneration of the central and peripheral nervous system, hypertrophic cardiomyopathy and increased incidence of diabetes. FA is caused by a GAA repeat expansion in the first intron of the gene encoding frataxin, an evolutionarily conserved mitochondrial protein, which results in decreased gene expression. Ubiquitous inactivation of the fly frataxin ortholog dfh blocks the transition from larval to pupal stages. In this study, we show that this phenotype is due to ecdysteroid deficiency and that feeding larvae with the 20-hydroxyecdysone steroid hormone rescues this developmental blockage. In mammals, adrenodoxin, the ferredoxin FDX1, is an Fe–S-containing protein essential for the synthesis of various steroid hormones. We show here that the two fly ferredoxins, Fdxh and Fdxh2 (encoded by CG1319), are also involved in steroidogenesis. This provides a potent mechanism by which frataxin, known to be involved in Fe–S cluster biosynthesis, could affect steroidogenesis through reduced ferredoxin activity. Finally, we show that frataxin inactivation decreases progesterone synthesis in human KGN ovarian granulosa cells. Thus, the involvement of frataxin in steroid synthesis appears to be a conserved function of the protein from flies to human and our data suggest that steroidogenesis could be affected in FA patients.

Introduction

Friedreich’s ataxia (FA) is the most frequent autosomal-recessive spinocerebellar ataxia in Caucasians with an incidence around 1/50 000. Neurological features of FA include spinocerebellar and sensory ataxia, dysarthria and pyramidal signs. Other features of the disease are muscle weakness, increased incidence in diabetes, scoliosis and hypertrophic cardiomyopathy. The first symptoms usually appear around puberty and lead to death with an average age around 40 years (1–4). FA is caused by a GAA trinucleotide repeat expansion in the first intron of the FXN gene encoding frataxin, an evolutionarily conserved mitochondrial protein. This results in decreased gene expression and decreased amount of the frataxin protein in the mitochondrial matrix (5). The frataxin cellular function is not so far fully characterized. Frataxin deficiency impairs iron–sulfur cluster (Fe–S) biosynthesis, and leads to decreases in aconitase and mitochondrial respiratory chain activities (6), hypersensitivity to oxidative stress (7) and accumulation of intra-mitochondrial iron associated with depletion of cytosolic iron in affected organs (8–10).

Thanks to the high degree of conservation of frataxin throughout evolution, several animal models of FA have been developed in mouse, flies and worms [reviewed in (11)]. All the Drosophila models of FA are currently based on RNAi-mediated down-regulation of dfh (the fly ortholog of frataxin) either ubiquitously or specifically in various cell types (12–18). Glial-specific inactivation of frataxin leads to some pre-adult lethality. Viable adults exhibit reduced lifespan, hypersensitivity to oxidative stress and altered lipid metabolism with an increase in free fatty acids, pathological...
features partially rescued by overexpression of a Drosophila apolipoprotein D ortholog (Glial lizarillo) (15). Frataxin inactivation in the peripheral nervous system leads to reduced adult lifespan, prevented by overexpression of catalase, an H₂O₂-scavenging enzyme (13). We have recently developed a Drosophila cardiac model of FA, in which frataxin is inactivated specifically in the fly heart (17). This model recapitulates defects of cardiac function observed in patients and mouse models of FA (10,19,20). The fly heart phenotype is fully rescued by expression of human frataxin, demonstrating that fly and human frataxins share similar functions, at least in cardiomyocytes. This model was used to evaluate the therapeutic potential of several candidate compounds and led to the identification of Methylene Blue as a highly efficient compound to prevent cardiac dysfunctions in frataxin-depleted hearts. Thus, these tissue-specific Drosophila models of FA have proved to be reliable to identify and characterize disrupted pathways caused by frataxin deficiency and to evaluate therapeutic interventions.

Besides tissue-specific fly models, Anderson et al. (12) described a developmental phenotype induced by ubiquitous inactivation of frataxin. In Drosophila, after hatching, larvae progress through three larval stages separated by a molt. At the end of the third larval stage, the pupariation marks the onset of metamorphosis. Ubiquitous inactivation of frataxin throughout development leads to a developmental blockage at the third larval stage. Frataxin-deficient larvae are unable to pupariate or reach pupariation later than controls, continue to grow and become giant (12). These larvae also present reduced activities of Fe–S-containing mitochondrial aconitase and of respiratory complexes II–IV, hypersensitivity to iron and altered lipid metabolism (12,15). In this work, we investigated the mechanisms by which frataxin deficiency may lead to this developmental phenotype. This study was stimulated by two aims. First, we postulated that it could reveal new physiological functions of frataxin potentially conserved and affected in the FA disease. Secondly, this characterization was a prerequisite to determine the relevance of using this phenotype for large-scale drug screening.

The timing of developmental transitions is coordinated in flies by the titers of the ecdysteroid hormone 20-hydroxyecdysone (20E). Indeed, a high titer pulse of 20E at the end of larval development is required to initiate the prepupal stage of development and to signal the onset of metamorphosis. Delayed pupariation and formation of giant larvae have been observed in several genetic contexts of ecdysteroid production deficiency in Drosophila (21–24). Thus, we hypothesized that frataxin inactivation could affect steroidogenesis in flies.

Here, we show that frataxin is required to produce ecdysteroids in the steroidogenic prothoracic gland and that 20E feeding rescues the defective transition from larval to pupal stages in frataxin-depleted flies. As frataxin is known to be involved in Fe–S synthesis, we hypothesized that frataxin deficiency could affect activities of Fe–S enzymes involved in steroidogenesis. Indeed, in mammals, the ferredoxin FDX1, also called adrenodoxin, is an Fe–S protein essential in steroid hormone biosynthesis, where it acts as an electron mediator between adrenodoxin reductase and several mitochondrial Cytochromes P450 (CYPs) [reviewed in (25)]. Here, we show that the two Drosophila ferredoxins, Fdxh and the protein encoded by CG1319 (mentioned below as Fdxh2), are also required for ecdysteroid synthesis, suggesting that these Fe–S cluster-containing enzymes could mediate the effect of frataxin inactivation on steroidogenesis. Finally, to determine if frataxin is also required for steroid synthesis in mammals, we used the KGN cell line, a steroidogenic human granulosa tumor cell line known to secrete progesterone (26).

In mammals, progesterone is the product of the two steps conversion of cholesterol, first to pregnenolone by CYP11A1 and subsequent to progesterone by 3β-hydroxysteroid dehydrogenase. FDX1 functions as the electron donor for the catalytic activity of CYP11A1 (27) and accordingly, FDX1 silencing in KGN cells significantly reduces progesterone secretion (28). Here, we observed that human frataxin knockdown also reduces progesterone secretion in KGN cells. Thus, the involvement of frataxin in steroid synthesis appears to be conserved from flies to humans.

Results
Frataxin ubiquitous inactivation leads to ecdysteroid deficiency in flies

We investigated whether frataxin inactivation affects the 20E pulse preceding pupariation. We inactivated dfh by RNA interference, using a UAS-fhRNAi construct combined to the ubiquitously expressed daGAL4 driver, similarly to experiments performed by Anderson et al. (12). In our breeding conditions (23°C), more than 85% of the control daGAL4+ larvae reached pupariation between 140 and 160 h after egg deposition, when only 3% of daGAL4>+UAS-fhRNAi formed pupae before 160 h. The frataxin-deficient pupae were not capable of becoming viable adults. We quantified the level of ecdysteroids in both types of larvae, at 140 h, with an enzymatic immunoassay. We used an ecdysteroid antiserum that detects ecdysone, 20E, and other ecdysteroid metabolites including 2-deoxyecdysone. Ecdysteroids were 71% lower in daGAL4>+UAS-fhRNAi larvae compared with daGAL4+ larvae (Fig. 1A).

To further confirm this steroid deficiency, we have followed the activation of the sgs3:GFP reporter, a fusion protein under the control of the sgs3 promoter. Sgs3 is a glue gene expressed in salivary glands under control of 20E (29,30). Under our breeding conditions, GFP in the salivary glands of daGAL4>+sgs3:GFP control larvae became detectable through the body wall at 130 h after egg deposition and the signal was strong from 135 h to the formation of prepupae (Fig. 1B). In daGAL4>+UAS-fhRNAi; sgs3:GFP frataxin-deficient larvae, GFP was undetectable before 190 h. These results suggest that frataxin-deficient larvae are not able to properly initiate the pupariation process because they fail to produce the ecdysteroid peak required to proceed to pupal stages.

20E feeding rescues the defective larval to pupal transition in frataxin-deficient larvae

To further demonstrate that the developmental delay is due to reduced levels of 20E, we performed 20E feeding experiments. Larvae were collected at 120 h after egg laying and transferred on a medium containing 500 µg/ml 20E or EtOH for control conditions. All the 20E-fed daGAL4>+UAS-fhRNAi larvae reached pupariation within 2 days after the transfer, with a timing of pupariation close to daGAL4+ control larvae. At 160 h, 85% of the 20E-fed larvae have formed pupae, compared with 3% for untreated larvae (Fig. 1C). However, the pupae rescued by 20E feeding were not able to develop further and died, maybe due to inappropriate levels of ecdysteroids during the pupal stage, where food-delivered 20E supplementation does not occur. Accordingly, 20E feeding also failed to drive metamorphosis into adulthood in other genetic contexts of steroid deficiency (24,31).

Frataxin is required in the fly prothoracic gland for the ecdysteroid synthesis and the larval to pupal transition

In Drosophila, the steroid hormone ecdysone is synthesized in the prothoracic gland, secreted in the hemolymph and converted to...
20E, its active form, in peripheral tissues. We investigated whether frataxin inactivation in the prothoracic gland was responsible for the defective larval to pupal transition observed previously. Expression of fh-RNAi was driven in the prothoracic by the phmGAL4 driver. At 23°C, phmGAL4>UAS-fhRNAi larvae presented delayed pupariation and 32% of the larvae failed to pupariate (Fig. 2A). Because GAL4 activity is temperature-dependent, we tested higher breeding temperatures. The phenotype was stronger at 26°C, where all the larvae failed to pupariate. These larvae survived at least 3 more weeks and became giant larvae (Fig. 2B). The next experiments were performed at 26°C. First, we quantified ecdysteroids at 140 h after egg laying with an enzymatic immunoassay (Fig. 2C). Ecdysteroids were 35% lower in phmGAL4>UAS-fhRNAi larvae compared with phmGAL4>+ larvae. We also followed the activation of the sgs3:GFP reporter. The GFP signal was undetectable in the salivary glands of phmGAL4>UAS-fhRNAi; sgs3:GFP larvae at 120 h, whereas it was strong in salivary glands of phmGAL4,sgs3:GFP-> (Fig. 2D). Then, we performed rescued experiments with 20E feeding, starting at 96 h. This treatment restored pupariation of phmGAL4>UAS-fhRNAi larvae, even leading to a slightly advanced pupariation compared with phmGAL4-> controls (Fig. 2E). The expression of sgs3:GFP was also restored in phmGAL4>UAS-fhRNAi; sgs3:GFP fed with 20E (Fig. 2D). Thus, frataxin is required in the prothoracic gland to produce the ecdysone peak required at the third instar for larval to pupal transition. Accordingly, we did not observe delayed pupariation when expression of the dfh RNAi was driven in other tissues, including salivary glands (lioGAL4 driver), fat body (pp1GAL4 driver), neurons (elavGAL4 and AppGAL4 drivers) and glial cells (repoGAL4 driver) (data not shown).

7-Dehydrocholesterol fails to efficiently rescue pupariation in frataxin-depleted larvae

Next, we investigated the mechanisms by which frataxin deficiency may lead to impaired ecdysone synthesis. Frataxin deficiency is known to affect the Fe–S cluster assembly.
Consequently, frataxin depletion could affect the activity of Fe–S-containing enzymes involved in steroid biosynthesis. In Drosophila, the first enzymatic step of ecdysone biosynthesis is the conversion of cholesterol to 7-dehydrocholesterol (7-DHC) by Neverland (Nvd), a Rieske/Fe–S-containing enzyme (Fig. 3A). The loss of Nvd function in the prothoracic gland causes arrest of larval molting and growth. When 7-DHC is added to the food, this phenotype is completely rescued and animals develop into adults (31,32). If frataxin deficiency exclusively affects Nvd activity and consequently the cholesterol to 7-DHC conversion in the ecdysone biosynthetic pathway, 7-DHC should rescue the defective larval to pupal transition in frataxin-depleted larvae. We treated phmGAL4>UAS-fhRNAi larvae with 7-DHC. At 23°C, 7-DHC weakly improved the timing of pupariation (Fig. 3B). However, the final percentage of larvae reaching pupariation was not improved. Moreover, 7-DHC treatment failed to rescue the complete absence of pupariation of phmGAL4>UAS-fhRNAi larvae at 26°C. Thus, steps of the ecdysone synthesis downstream of 7-DHC are likely to be also affected in frataxin-depleted larvae.

Figure 2. Frataxin is required in the prothoracic gland for ecdysone synthesis and the larval–pupal transition. (A) Timing of pupariation at 23°C and 26°C of phmGAL4>UAS-fhRNAi and UAS-fhRNAi+ larvae (23°C, n = 434; 26°C, n = 112). (B) Representative phmGAL4+ and phmGAL4-UAS-fhRNAi individuals. Times after egg laying are indicated. Scale bar: 1 mm. (C) Levels of ecdysteroids in phmGAL4+ and phmGAL4-UAS-fhRNAi larvae, collected 140 h after egg laying (8 samples of 15 individuals for phmGAL4+, 8 samples of 15 individuals for phmGAL4-UAS-fhRNAi larvae). Values are means ± SEM. Significant difference with the phmGAL4+ control is shown (*P < 0.05). (D) Sgs3:GFP expression at 120 h after egg laying in phmGAL4-+; sgs3:GFP and phmGAL4-+; sgs3:GFP-UAS-fhRNAi larvae treated or untreated with 20E. 20E treatment started from 96 h after egg laying and larvae were imaged at 120 h. (E) Timing of pupariation following 20E feeding. phmGAL4+ and phmGAL4-UAS-fhRNAi larvae were transferred at 96 h on medium supplemented with EtOH (phmGAL4+ EtOH, n = 36; phmGAL4-UAS-fhRNAi EtOH, n = 30) or 20E (phmGAL4-UAS-fhRNAi 20E, n = 30).
Fdxh and Fdxh2, the two fly ferredoxins, are required for ecdysone synthesis

The biosynthetic pathway from cholesterol to 20E is not fully characterized. The genes phantom (phm), disembodied (dib), shadow (sad) and shade (shd) encode cytochrome P450 (CYP) enzymes that catalyze the final four hydroxylation steps (Fig. 3A) (33–36). Blast search revealed two genes encoding ferredoxins in Drosophila melanogaster, Fdxh and CG1319. Humans also possess two ferredoxins, the adrenodoxin FDX1, highly expressed in the adrenal gland and kidney cells, and the ubiquitously expressed FDX2 (37). FDX1, but not FDX2, is required for efficient conversion of 11-deoxycortisol in cortisol by CYP11B1 in vitro and is thought to be specifically required for steroidogenesis (37). FDX2 is involved in Fe–S cluster assembly and whether this is a specific function of this ferredoxin or a function shared with FDX1 is under debate (37–39). Phylogenetic analysis revealed that the fly Fdxh is more closely related to FDX2, when Fdxh2 (Q8SZA8, encoded by CG1319) is more closely related to FDX1 (40). Whether Fdxh and Fdxh2 are required for ecystoder synthesis in flies was unknown. However, the involvement of at least one of these proteins in steroidogenesis is suggested by the fact that null mutants of dare, the gene encoding adrenodoxin reductase, undergo developmental arrest that can be rescued by 20E feeding (41). We inactivated ubiquitously Fdxh and Fdxh2 by RNAi using the daGAL4 driver. Ecdysteroids were 87% lower in daGAL4>UAS-FdxhRNAi larvae compared with daGAL4>+ larvae of the same age (Fig. 4A). Moreover, these larvae presented delayed pupariation or failed to pupariate, a phenotype fully rescued by 20E feeding (Fig. 4B). Then, we inactivated Fdxh in the prothoracic gland and observed similarly delayed or absent pupariation with formation of giant larvae (Fig. 4C), a developmental phenotype also rescued by 20E feeding (Fig. 4D). Thus, Fdxh is required for steroidogenesis in the prothoracic gland and Fdxh inactivation phenocopied frataxin inactivation.

Fdxh2 ubiquitous inactivation, in contrast with Fdxh inactivation, led to larval growth defects and strong larval lethality. Only 55% of daGAL4>UAS-Fdxh2RNAi first instar larvae reached the third larval stage, quantified at 120 h of development (Fig. 5A). In contrast, with daGAL4>UAS-FdxhRNAi larvae, which remained alive and formed giant larvae, these larvae died progressively, essentially in the following 5 days, and <1% formed pupae (Fig. 5A). daGAL4>UAS-Fdxh2RNAi larvae also presented growth defects during the third larval stage: their size was similar to controls at 96 h but they were smaller than controls at 120 and 140 h (Fig. 5B). At 150 h, their mean larval weight was 0.57 mg (±0.02, n = 450) compared with 1.83 mg (±0.09, n = 180) for daGAL4>+ control larvae of the same age. We measured ecdysteroids in these larvae, which were 83% lower compared with control larvae (Fig. 5C). Then, we inactivated Fdxh2 specifically in the prothoracic gland with the phmGAL4 driver. This also led to larval growth defects and lethality at the third larval stage, with a
complete lack of pupariation (Fig. 5B and Supplementary Material, Fig. S2B). We conclude from these experiments that Fdxh2 is required in the prothoracic gland for steroidogenesis and normal development. However, 20E feeding failed to rescue the defective pupariation associated with Fdxh2 ubiquitous inactivation and even accelerated the larval death (Supplementary Material, Fig. S2A). When Fdxh2 was inactivated specifically in the prothoracic gland, we only observed a weak rescue of pupariation with 9% of the 20E-fed larvae that formed pupae (Supplementary Material, Fig. S2B). This lack of rescue by 20E feeding could be due to irreversible developmental defects induced by Fdxh2 inactivation at earlier stages of development. Accordingly, we observed tracheal defects in many daGAL4>UAS-Fdxh2RNAi larvae, with signs of necrosis (Fig. 5D). Similar tracheal defects have been observed in DHR3 and DHR78 mutants, two ecdysone-induced nuclear receptors required during the larval molts to control the molting and patterning of the tracheal cuticle (42,43). Therefore, our data suggest that Fdxh2 inactivation leads to ecdysteroid deficiency occurring earlier than in the case of Fdxh inactivation, affecting the larval molts leading to larval lethality at the third larval stage.

However, Fdxh2 inactivation could also lead to other mitochondrial defects besides deficient steroidogenesis that could participate to the larval lethality. Noticeably, based on the recent reports on human FDX2 involvement in Fe–S cluster assembly (37–39), we wondered if Fdxh2 and/or Fdxh could have a similar function. In agreement with this hypothesis, the phenotypes induced by ubiquitous inactivation of Fdxh2, namely the larval growth arrest and lethality, were similar to those induced by Hsc20 mutations, a protein involved in Fe–S cluster assembly (44). Therefore, we quantified the enzymatic activity of the succinate dehydrogenase Fe–S protein in third instar larvae ubiquitously depleted for Fdxh and Fdxh2. At 150 h, succinate dehydrogenase activity per mitochondrial protein weight was not decreased by Fdxh inactivation (Supplementary Material, Fig. S3), and was unexpectedly significantly increased compared with control larvae following Fdxh2 inactivation. Further investigations are now required to determine if these two fly ferredoxins are effectively not required for Fe–S cluster assembly or alternatively if they share this function, explaining why inactivation of a single ferredoxin did not impact Fe–S protein activity.

In summary, we observed that the fly ferredoxins Fdxh and Fdxh2 are required for ecdysteroidogenesis in flies, suggesting that these two Fe–S enzymes could mediate the effect of frataxin inactivation on steroidogenesis.

Frataxin is required for progesterone synthesis in a human cell model of granulosa

Next, we wanted to test a potential implication of human frataxin in steroid hormone synthesis and secretion. In mammals,
progesterone is an endogenous steroid hormone produced mainly by the corpus luteum in the ovary and, to a lesser extent, by granulosa cells at the end of the follicular development.

We investigated if frataxin inactivation affects progesterone secretion in the cell line KGN, a human granulosa tumor cell line known to secrete progesterone (26). We inactivated frataxin by siRNA transfection and examined the effect on progesterone production. First, we checked the efficiency of siRNA FXN knockdown by RT-qPCR and western blotting. Transfection of siRNA against FXN significantly knocked down the expression of FXN in KGN cells, with an FXN residual mRNA level ~16% of control cell level (Fig. 6A), and a strong decrease in frataxin protein level (Fig. 6B). The amount of progesterone in the culture media was then measured by radio immunoassay. Reducing the level of the frataxin protein strongly attenuated progesterone secretion in KGN cells, which was 66% lower than in control cells (Fig. 6C).

**Discussion**

There is increasing consensus on the involvement of frataxin in the mitochondrial Fe–S cluster biosynthesis machinery (45–50).
Fe–S clusters are prosthetic groups essential for enzymatic activities involved in many fundamental processes. Frataxin inactivation is thus susceptible to affect a large number of cellular and physiological functions. We have shown here that frataxin inactivation affects steroidogenesis both in flies and in human ovarian cells. Thus, the involvement in steroid hormone synthesis appears to be a conserved function of the frataxin protein. This may appear surprising at first glance considering that steroid hormone structures, sites of synthesis and physiological roles are different from flies to humans. However, mechanisms of steroid hormone synthesis share common features, in particular the involvement of several microsomal and mitochondrial CYPs [reviewed in (51,52)]. In mammals, FDX1 is required to transfer electrons from an NADPH-dependent ferredoxin reductase to mitochondrial class I CYPs involved in steroid synthesis [reviewed in (53)]. Here, we show that the fly ferredoxins, Fdxh and Fdxh2, are also required to produce ecdysteroids, providing an additional common feature with mammalian steroid hormone synthesis.

We investigated the mechanisms by which frataxin deficiency affects ecdysteroid titters in flies. Ecdysone is synthesized in the prothoracic gland from cholesterol or plant sterols provided in the diet, and the conversion to its active form 20E occurs in peripheral tissues by the shade cytochrome P450, expressed notably in the fat body and in the gut (54). Frataxin inactivation specifically in the prothoracic gland is sufficient to decrease the 20E titer, showing that ecdysone synthesis is altered in this steroidogenic gland. This hormonal defect is not only due to decreased activity of the Fe–S-containing enzyme Nvd, which converts cholesterol into 7-DHC, since 7-DHC failed to rescue delayed pupariation of frataxin-depleted larvae at 26°C, albeit it slightly improved the phenotype at 23°C. Thus, steps downstream of Nvd, from 7-DHC to ecdysone, are affected by frataxin inactivation. This part of the biosynthetic pathway is not fully characterized, in particular the steps from 7-DHC to 5β-ketodiol, referred as the so-called ‘black box’, but the following hydroxylation steps are catalyzed by cytochrome P450s encoded by members of the Halloween genes, phm, dib and sad [reviewed in (55)].
While Phm is a microsomal P450, Db and Sad are mitochondrial. We have shown here that the fly ferredoxins Fdxh and Fdxh2 are required for ecdysone synthesis. Thus, we propose that frataxin inactivation affects the activities of the Fe–S protein Nvd and of the ferredoxins Fdxh and Fdxh2 and consequently, the activities of the mitochondrial Dib and Sad cytochrome P450s. However, we cannot exclude that other mitochondrial dysfunctions induced by frataxin inactivation participate to the steroid deficiency.

The phenotypes induced by Fdxh2 inactivation were more severe compared with Fdxh inactivation. Since 20E feeding failed to rescue the defective pupariation, it was unclear whether these phenotypes, in particular the larval defective growth and lethality, were exclusively due to steroid deficiency. 20E feeding experiments at earlier stages are required to clarify this point, similarly to the experiments performed to rescue the larval lethality induced by Nvd inactivation (31). The developmental blockage due to Fdxh inactivation occurs only at the end of the third larval stage. This suggests that Fdxh2 expression in the prothoracic gland is sufficient in earlier larval stages to insure the production of the ecdysone peaks controlling the larval molts from the first to the second, and the second to the third larval stages. Fdxh would in this case only be required for the massive production of ecdysone at the end of the third larval stage preceding pupariation. Alternatively, the two ferredoxins may have different temporal expression patterns in the prothoracic gland explaining why they are required at different developmental time points. It should be reminded that RNAi only leads to partial inactivations, which complicates the comparison of the phenotype strengths.

Human FDX2 and the yeast Yah1 ferredoxin are required for Fe–S protein biogenesis (37–39,56). Here, we did not observe decreased activity of the Fe–S protein succinate dehydrogenase due to Fdxh or Fdxh2 inactivation. This enzymatic activity was even increased in larvae depleted for Fdxh2 when compared with control larvae of the same age. This unexpected finding could be due to the developmental blockage and defective growth of these larvae which could maintain them in a state of higher metabolism compared with control, aged matched, third instar wandering larvae. Thus, the succinate dehydrogenase enzymatic activity should be tested at earlier time points, when the developmental blockage has not yet occurred. Further investigations are required to determine if the fly ferredoxins are involved in Fe–S cluster assembly. If this function is shared by the two ferredoxins, the analysis of double mutants will be also required. Such interesting studies are out of the scope of this paper which focused on frataxin function.

The involvement of frataxin in steriodogenesis appears to be evolutionarily conserved. Indeed, we demonstrated in this study that FXN inactivation affects progesterone synthesis in human ovarian KGN cells. This effect could similarly be due to decreased ferredoxin activity. Accordingly, FDX1 inactivation in KGN cells affects progesterone synthesis in a similar way (28) and ferredoxin inactivity is reduced in frataxin-deficient oligodendroglioma cells (57). Ferredoxins are involved in numerous metabolic processes, including Fe–S biogenesis, bile acid synthesis, vitamin D metabolism and steroidogenesis (25,37,38). In particular, FDX1 provides electron for CYP11A1, which catalyzes hydroxylation steps that convert cholesterol to pregnenolone, the precursor of all mammalian steroid hormones, and for CYP11B1 and CYP11B2, which catalyze the final steps in the biosynthesis of cortisol and aldosterone. Apart from steroidogenic CYPs, CYP2A41, CYP27A1 and CYP27B1, key enzymes of vitamin D3 metabolism also receive electrons from FDX1 (58). Therefore, frataxin inactivation, through potential decreased ferredoxin activity, is susceptible to lead to multiple hormonal imbalances besides the effect on progesterone observed in this study.

We conclude that steriodogenesis is likely to be altered in FA. Such deficiencies have not been described so far in patients, most likely excluding strong hormonal defects. However, mild undiagnosed impairments could be present and contribute to some extent to the clinical features of the disease. This could explain, at least in part, why the first symptoms of the disease usually appear around puberty. Progesterone is synthesized in ovariies, placenta and adrenal glands but also within the nervous system, in both neuronal and glial cells. Progesterone and its neuroactive metabolites play numerous physiological roles in the adult nervous system and have been shown to exert neuroprotective effects in several contexts of neurological diseases [reviewed in (59,60)]. Thus, to investigate whether progesterone and more generally steroid hormone synthesis are affected in FA patients is an important issue. However, most hormones including steroids are well known to show strong circadian and individual variations in their concentrations in normal individual. In addition, the first symptoms of the FA disease usually appear around puberty, a period during which steroid concentrations undergo dynamic changes. Thus, we believe that it will be necessary to perform steroid assays on a large cohort of patients in a standardized and dedicated clinical protocol, with age- and sex-matched controls. We hope that our present work will impulse such studies to evaluate the potential relevance of steroid hormone therapy in FA.

**Materials and Methods**

**Dfh stocks and culture methods**

UAS-fhRNAi (w[1]; Pw[+mc]=UAS-fh.IR2), w[118], sgs3:GFP (w); P(Sgs3-GFP)[3], da-GAL4 (P[da-GAL4-da.G32]), elavGAL4 (P[elavGAL4-elav[C155]], ppGAL4 (w); P[elavGAL4-P2]), and repoGAL4 (w[118]; P[elavGAL4repo/TM3, Sb[1]]) were obtained from the Bloomington Stock Center. UAS-Fdxh2RNAi (P[KK109392]VIE-260B) and UAS-Fdxh2RNAi (P[KK102957]VIE-260B) were obtained from the VDRD Stock Center. phmGAL4 (w; UAS-mCD8-GFP[II]); phmGAL4 (III)/SM5-TM6B) and lioGAL4 (w; Lio GAL4/Cyo-GFP; Ly/TM6 Tb Sb) were kindly provided by J. Montagne and F. Perronet, respectively. For developmental timing measurements, phmGAL4 females (w; UAS-mCD8-GFP[II]); phmGAL4 (III]/SM5-TM6B) were crossed with UAS-fhRNAI males. In the progeny, phmGAL4-UAS-fhRNAi individuals were identified by the lack of the SM5-TM6B balancer chromosome. UAS-fhRNAI->SM5-TM6B individuals issued from the same cross (referred as UAS-fhRNAI->+) were used as control larvae.

**Ecdysteroid measurements**

Ecdysteroid measurements were performed with competitive Enzyme Immunoassay (EIA) (Cayman Chemicals, Inc., USA) as previously described by Hackney et al. (61). The standard curve was obtained with 20E (Sigma-Aldrich), and thus the results are expressed in 20E equivalents. For sample preparation, larvae were weighed (15 larvae per sample, 45 larvae for daGAL4->UAS-Fdxh2RNAi) homogenized in 300 µl of methanol and centrifuged (20 min at 18 000g). The supernatant was dried and resuspended in 50 µl of EIA Buffer prior to the EIA assay. Ellmann reagent (Cayman Chemicals, Inc.) was used for the chromogenic reaction and absorbance was read at 415 nm on a FlexStation 3 (Molecular Devices).

**Statistical significance** was assessed by non-parametric Wilcoxon analysis.

**20E and 7-DHC feeding experiments**

For 20E feeding, third instar larvae were collected and placed in groups of 20 individuals in tubes containing medium
supplemented with 20E (Sigma-Aldrich) at a final concentration of 500 µg/ml, from a 20E stock solution at 10 mg/ml in EtOH, or medium supplemented with EtOH for control larvae.

For 7-DHC feeding experiments, standard medium was supplemented with 7-DHC (Sigma-Aldrich) at a final concentration of 60 µg/ml, from a 7-DHC stock solutions at 5 mg/ml in EtOH. These experiments were carried out under constant dark conditions because 7-DHC is unstable in light.

**Cell culture transfection of siRNA and measurement of progesterone**

The human granulosa tumor cell cell line KGN (26) was purchased from the Riken Resource center (RRBC-RCB1154, Riken Cell Bank, Ibaraki, Japan) after approval by Dr Yoshiro Nishi and Dr Toshihiko Yanase. KGN cells were maintained in DMEM/F-12 supplemented with 5% charcoal-stripped FBS. Each transfection was done in triplicate in two independent

Western blot

Proteins of siRNA-transfected KGN were resuspended in Laemmli buffer after Trizol extraction. Protein concentration was estimated using the Pierce BCA Protein Assay Kit and equal aliquots were electroporated (one pulse, 30 ms, 1.5 kV) and seeded into a well of 24-well dish in 500 µl DMEM/F-12, no phenol red, supplemented with 7-DHC (Sigma-Aldrich) at a final concentration of 60 µg/ml, from a 7-DHC stock solutions at 5 mg/ml in EtOH. These experiments were carried out under constant dark conditions because 7-DHC is unstable in light.

**Western blot**

Proteins of siRNA-transfected KGN were resuspended in Laemmli buffer after Trizol extraction. Protein concentration was estimated using the Pierce BCA Protein Assay Kit and equal amounts of lysates (40 µg) were used for immunoblotting. Samples were resolved on a 16% SDS–polyacrylamide gel and then transferred to a nitrocellulose membrane (Amer sham Proran, GE Healthcare) by electroblotting. After blocking with 5% non-fat dry milk, the blot was incubated with anti-FXN mouse monoclonal antibody (ab110328, Abcam) or anti-GAPDH (14C10, Cell Signaling) as control and was developed with AP-conjugated secondary antibodies using a chemiluminescent substrate.

**Supplementary Material**

Supplementary Material is available at HMG online.

**References**


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