Humans possess two mitochondrial ferredoxins, Fdx1 and Fdx2, with distinct roles in steroidogenesis, heme, and Fe/S cluster biosynthesis

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Mammalian adrenodoxin (ferredoxin 1; Fdx1) is essential for the synthesis of various steroid hormones in adrenal glands. As a member of the [2Fe-2S] cluster-containing ferredoxin family, Fdx1 reduces mitochondrial cytochrome P450 enzymes, which then catalyze; e.g., the conversion of cholesterol to pregnenolone, aldosterone, and cortisol. The high protein sequence similarity between Fdx1 and its yeast adrenodoxin homologue (Yah1) suggested that Fdx1, like Yah1, may be involved in the biosynthesis of heme A and Fe/S clusters, two versatile and essential protein cofactors. Our study, employing RNAi technology to deplete human Fdx1, did not confirm this expectation. Instead, we identified a Fdx1-related mitochondrial protein, designated ferredoxin 2 (Fdx2) and found it to be essential for heme A and Fe/S protein biosynthesis. Unlike Fdx1, Fdx2 was unable to efficiently reduce mitochondrial cytochromes P450 and convert steroids, indicating that the two ferredoxin isoforms are highly specific for their substrates in distinct biochemical pathways. Moreover, Fdx2 deficiency had a severe impact, via impaired Fe/S protein biogenesis, on cellular iron homeostasis, leading to increased cellular iron uptake and iron accumulation in mitochondria. We conclude that mammals depend on two distinct mitochondrial ferredoxins for the specific production of either steroid hormones or heme A and Fe/S proteins.

adrenodoxin | cytochrome P450 | iron | iron-sulfur cluster | IRP1

The ferredoxin family comprises iron-sulfur (Fe/S) proteins whose evolutionary debut presumably predates membrane-bound life (1). Similar to many other Fe/S proteins, ferredoxins exploit the redox properties of their [2Fe-2S] or [4Fe-4S] cofactors to transfer electrons in a wide variety of biochemical reactions throughout numerous, divergent species. The subfamily of [2Fe-2S] ferredoxins is of bacterial origin, has a molecular mass of 6–25 kDa and is negatively charged at neutral pH. The most well-known eukaryotic members of this subfamily are present in plastids of plants or algae and in mitochondria. Various isoforms of plant (2) and algal (3) ferredoxins were recently demonstrated to perform diverse and highly substrate-specific activities. These ferredoxins differ substantially in both protein sequence and function from mitochondrial and bacterial [2Fe-2S] ferredoxins that are highly related in sequence. In mammalian mitochondria, adrenodoxin (Adx; herein termed Fdx1) participates in adrenal steroidogenesis, bile acid, and vitamin D synthesis (see refs. 4–7 for review). In these biochemical pathways, electrons are transferred from NADPH via Adx reductase (AdR; FdxR) to Fdx1, which in turn reduces members of the mitochondrial cytochrome P450 (CYP) protein family such as P450scc (CYP11A1) and the CYP11B subgroup. The former enzyme catalyzes the production of pregnenolone, the rate-limiting step in adrenal steroid biosynthesis, whereas CYP11B enzymes convert later intermediates into cortisol, corticosterone, or aldosterone.

Bacterial and mitochondrial [2Fe-2S] ferredoxins perform a basic biosynthetic function in the maturation of Fe/S proteins (8–11). The yeast adrenodoxin homologue 1 (Yah1) receives its electrons from Arh1 (yeast adrenodoxin reductase homologue 1), which uses NADH as an electron source (12–14). Arh1 and Yah1 function in an early step of Fe/S protein biogenesis, the assembly of a transient Fe/S cluster on the Fe/S scaffold protein Isu1. An additional function of mitochondrial Yah1 has been demonstrated by studies initially carried out in yeast: the formation of the heme A cofactor of cytochrome *c* oxidase (respiratory complex IV [COX]) (15, 16). In this process, Arh1 and Yah1, together with the heme A synthase Cox15, participate in the hydroxylation of heme O to heme A, which is then assembled into COX. The ferredoxin electron transfer chains are of considerable medical importance. A genetic defect in human Cox15 leads to infantile COX deficiency and cardiomyopathy, AdR has been shown to act as a tumor suppressor, and defects in CYPs are associated with several diseases (17–19).

DNA sequence analysis of the human genome for homologues of yeast Yah1 yields two such genes: *FDX1* (chromosome 11q22) and *FDX2* (19p13.2; annotated as "Fdx1L"). Protein sequence alignment reveals that the predicted, mature form of Fdx2 has 50% identity and 73% similarity with Yah1, whereas those of Fdx1 and Yah1 are 48% and 73%, respectively; Fdx1 and Fdx2 share 43% identity and 69% similarity (cf. ref. 20). It is generally assumed from the high sequence similarity of mammalian adrenodoxin and yeast Yah1 that the former protein is also capable of catalyzing the biosynthesis of Fe/S clusters and heme A (9, 10, 13, 15). In the current study, we tested this assumption for human adrenodoxin (Fdx1). Our results indicate that the expression and function of Fdx1 is largely confined to the adrenal gland and has no detectable general role in mammalian Fe/S protein biogenesis. Instead, we identify a heretofore uncharacterized, human [2Fe-2S] cluster ferredoxin (henceforth referred to as ferredoxin 2; Fdx2). Like Fdx1, Fdx2 is localized to mitochondria, contains a [2Fe-2S] cluster, and can accept electrons from NADPH via FdxR. Importantly, Fdx2 is essential for Fe/S protein biogenesis in human cells and can functionally replace Yah1. However, unlike Fdx1, Fdx2 is unable to reduce mitochondrial cytochrome P450. Thus, despite their striking sequence similarity, human Fdx1 and Fdx2 show a remarkable specificity for distinct biochemical processes.

Results

Fdx2 Is a Mitochondrial [2Fe-2S] Protein. To examine whether Fdx2 is expressed in human cells and imported into mitochondria, we

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raised antibodies against recombinant, His-tagged Fdx2. We then analyzed subcellular fractions generated from human HeLa cells by immunoblotting. A protein cofractionating with a mitochondrial marker (α/β subunits of F_1-ATPase) and with an apparent molecular mass of 22 kDa was recognized by our anti-Fdx2 antibody (Fig. 1*A*).

To compare the relative expression levels of the two ferredoxins in HeLa cells, we used a dilution series of recombinant purified proteins for immunoblotting. Fdx1 and Fdx2 were present in HeLa cells at 90 and 40 ng/mg cell protein, respectively. We then analyzed the relative abundance of these proteins in various human tissues. Fdx1 protein was undetectable in most tissue lysates, but was highly expressed in the adrenal gland, and present in kidney and testes (Fig. S1). This expression pattern fits perfectly with the function of Fdx1 in steroidogenesis, but is inconsistent with its predicted function in Fe/S protein biogenesis. In contrast, Fdx2 was detectable in virtually all tissues analyzed with the highest amounts present in testis, kidney, and brain.

We next asked whether Fdx2 is an Fe/S protein. Recombinant Fdx2 purified from *Escherichia coli* was red-brown in color and exhibited a UV-Vis spectrum characteristic of a [2Fe-2S] protein and remarkably similar to that of purified Fdx1, with an undulating, reduction-sensitive absorption shoulder between 400 and 500 nm (21) (Fig. 1*B*). The presence of a [2Fe-2S] cluster on Fdx2 was supported by EPR spectroscopy of dithionite-reduced protein (Fig. 1*C*). The axial EPR spectra of Fdx1 and Fdx2 had similar *g* values (2.02, 1.94) and were both detectable up to 80 K.

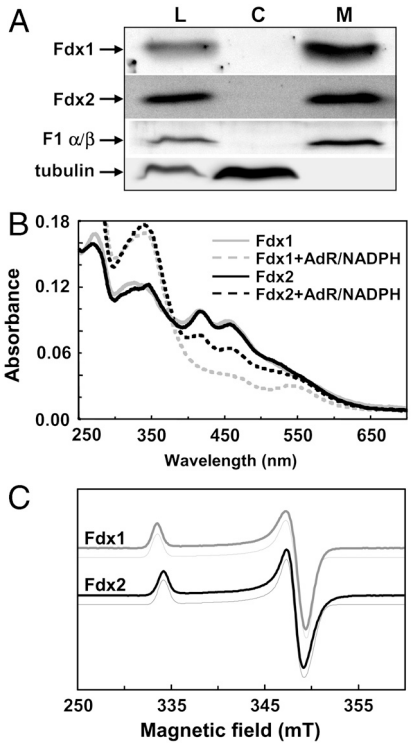

Fig. 1. Fdx2 is a mitochondrial [2Fe-2S] protein. (*A*) HeLa cell fractions, total lysate (L), cytosolic (C), and mitochondria-rich pellet (M), were evaluated by immunoblotting using antiserum against Fdx1 or Fdx2 as indicated. Probing with antibodies against F_1 ATPase α/β subunits or tubulin served as controls for mitochondrial and cytosolic loading, respectively. (*B*) Recombinant Fdx1 or Fdx2 (10 μM) were examined by UV-Vis spectroscopy before and after reduction by 0.1 μM AdR/2.5 mM NADPH. (*C*) EPR spectra (thick lines) and their simulations (thin lines) of dithionite-reduced recombinant ferredoxins. EPR conditions: microwave frequency, 9.458 GHz; microwave power, 0.013 mW; modulation frequency, 100 kHz; modulation amplitude, 1.25 mT; temperature, 10 K. Simulation parameters (g_z, g_y, g_x, and their Gaussian linewidths in mT): Fdx1: [2.0248, 1.9409, 1.9328, 1.08, 1.43, 1.27]; Fdx2: [2.0216, 1.9413, 1.9329, 1.12, 1.23, 1.67].

We also determined the reduction potentials of Fdx1 and Fdx2 to be -267 ± 5 mV and -342 ± 5 mV, respectively (22). In summary, these data indicate that Fdx2 is a mitochondrial [2Fe-2S] protein ubiquitously expressed in human tissues.

Fdx2, and Not Fdx1, Is a Member of the Mitochondrial Fe/S Protein Assembly Machinery. To investigate the function of Fdx2, the protein was depleted in HeLa cells using RNAi technology. To this end, cells were transfected with a pool of modified siRNAs that targeted three distinct regions of the mRNA. The transfections were repeated thrice, once every 3 d, and a final harvest of the cells was performed 3 d after the third transfection (i.e., day 9; cf. ref. 23). Fdx1 was analyzed in parallel. Immunoblotting against Fdx1 or Fdx2 verified an extensive depletion of the individual proteins over the course of the experiment (Fig. 2*A*).

Depletion of Fdx2 by RNAi caused a strong decrease in cell proliferation assigning an important function to Fdx2 in HeLa cells (Fig. 2*B*). In contrast, no significant effect was observed upon Fdx1 depletion. We next performed a series of enzyme assays to evaluate the results of Fdx1 or Fdx2 knockdowns on the activities of several Fe/S proteins. As depicted in Fig. 2*C*, the catalytic activities of mitochondrial and cytosolic aconitases, complex I, COX, and succinate dehydrogenase (SDH) relative to control proteins were all severely (65–90%) decreased upon Fdx2 depletion. This effect escalated with each subsequent transfection. In contrast, Fdx1 depletion had no effect on either aconitase isoform or complex I activity, and elicited a relatively weak (10–30%) diminishment of COX and SDH activities. More cycles of transfection did not augment these effects of Fdx1 deficiency. By transfecting HeLa cells separately with the individual siRNAs against Fdx1, we found that all three individual siRNA sequences elicited the same response as the siRNA pool, suggesting that the slightly dimished activities of COX and SDH were indeed the result of lowered Fdx1 levels. Mitochondria of Fdx2-deficient, but not of Fdx1-deficient cells exhibited a substantial loss of cristae membranes (Fig. S2), consistent with the severe effects on respiratory chain function upon Fdx2 depletion (23, 24).

Because Fdx1 and Fdx2 are highly homologous, we investigated whether they may have coinciding functions by testing if Fdx1 can replace Fdx2 function. Cells were thrice cotransfected with the Fdx2 siRNAs along with a vector encoding EGFP or the fusion proteins Fdx1-GFP (pFDX1) and Fdx2-GFP (psmFDX2). Silent mutations (sm) were introduced into the latter construct to avoid RNA interference. The strong effects of Fdx2 depletion on the two aconitases, SDH, COX, complex I, and mitochondrial ultrastructure were almost fully restored by cotransfection with psmFDX2 indicating the specificity of the RNAi approach (Fig. 2*D*, Fig. S2, and Fig. S3). No restoration of the enzyme activities was seen upon overexpression of Fdx1, indicating that Fdx1 cannot generally assume the role of Fdx2 in Fe/S protein biogenesis.

Estimating the protein amounts of several Fe/S proteins is also informative with respect to a cell's capacity to form the metallo-cofactor (25, 26). In line with the aforementioned results, the levels of mitochondrial aconitase, cytosolic aconitase (IRP1) and SDH were all decreased under Fdx2 depletion (Fig. 2*E*). Again, this defect was restored by cotransfection with psmFDX2, but not with pFDX1. In addition, an integral subunit (NDUFS3) of complex I, which harbors eight Fe/S clusters, ferrochelatase (FC; one [2Fe-2S] cluster) and glutamate phosphoribosylpyrophosphate amidotransferase (GPAT; one [4Fe-4S] cluster) were all specifically diminished by Fdx2 depletion relative to tubulin (loading control; Fig. 2*E*). As an additional strategy to detect alterations in respiratory complexes, we measured the radiolabelling of these complexes in cells that were treated with ^{55}Fe-transferrin for the 3 d following the third transfection. Analysis by blue native-polyacrylamide gel electrophoresis (BN-AGE) and autoradiography (24, 27) revealed that Fdx2 deficiency led to decreased ^{55}Fe incor-

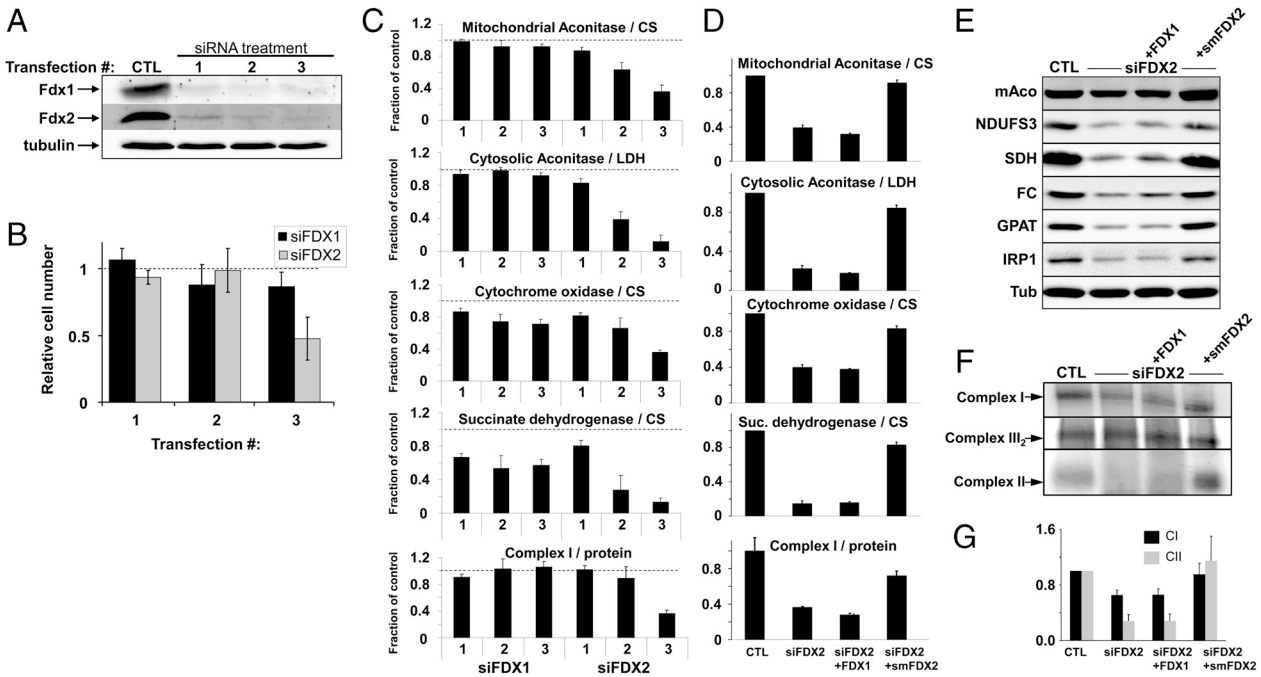

Fig. 2. Fdx2 is an essential component of the Fe/S protein biogenesis pathway in humans. (*A*) Depletion of the indicated ferredoxins by RNAi was verified by subjecting total HeLa cell lysates to immunoblotting following each of three sequential transfections with the respective siRNA. CTL, mock transfected cells; numbers indicate number of transfections. (*B*) HeLa cell growth after the transfections with Fdx-specific siRNAs (siFDX) was estimated by cell counting, and normalized to that of mock-transfected cells. (*C*) Enzyme activities of cell fractions from part B. The values were normalized to citrate synthase (CS) or LDH activities and displayed as the fraction of the control values for each transfection (dotted line). (*D*) Cells were cotransfected with the pEGFP-N3 vector (CTL) alone or together with the Fdx2 siRNA (siFDX2) and pFDX1 (encodes Fdx1-GFP; +FDX1) or, psmFDX2 (encodes Fdx2-GFP and contains silent mutations conferring RNAi avoidance; +smFDX2). Enzyme assays were performed as in (*C*) using the triply transfected samples. (*E*) Immunoblots from cells transfected as in part D, using the indicated antibodies. mAco, mitochondrial aconitase; NDUFS3, subunit of complex I; FC, ferrochelatase; GPAT, glutamate phosphoribosylpyrophosphate amidotransferase; IRP1, iron regulatory protein 1; Tub, tubulin. (*F*) Cells were transfected as in (*D*), and treated with 0.5 μM ^{55}Fe-transferrin after the third transfection. Crude mitochondrial fractions were subjected to blue-native PAGE, followed by phosphorimaging. (*G*) Densitometric analysis of the complexes I (CI) and II (CII) bands from three independent experiments as in part F. Error bars represent standard deviations.

poration into complexes I and II, which was restored by expression from plasmid psmFDX2 but not pFDX1 (Fig. 2*F*). The lack of an effect on complex III may be explained by dissociation of the supercomplex of complexes I, III, and COX upon diminishment of complex I (24) and the fact that half of the iron present in CIII is in heme. Taken together, the data presented in Fig. 2 strongly suggest a major role of Fdx2, but not of Fdx1, in Fe/S protein biogenesis.

Human Fdx2 Can Perform the Biochemical Functions of Yeast Yah1. We asked whether human Fdx2 can functionally replace yeast Yah1 in its tasks in Fe/S protein and heme A biosynthesis. We used a yeast strain (Gal-YAH1), in which a *GAL1-10* promoter has been inserted upstream of the *YAH1* coding region, thus permitting regulated expression of the *YAH1* gene (12). Gal-YAH1 cells grow at wild-type rates in galactose-containing media, yet exhibit a severe growth defect upon substituting glucose or glycerol for galactose. Expression and localization of the human ferredoxins, to which fungal mitochondrial targeting sequences were fused, was verified by immunoblotting (Fig. 3*A*). Human Fdx2 was able to restore growth of Gal-YAH1 cells on media containing either glucose or the nonfermentable carbon source glycerol (Fig. 3*B*). Consistent with a previous study, Fdx1 expression failed to replace Yah1 (28).

To analyze if Fdx2 can replace all functions of Yah1, we analyzed several biochemical parameters associated with functional loss of Yah1, including defective Fe/S protein biogenesis, heme biosynthesis, and iron regulation (29, 30). First, expression of Fdx2 restored the activity of the Fe/S protein aconitase to the same levels as yeast Yah1 (Fig. 3*C*). Second, Fdx2 was able to complement the defect in heme synthesis (29, 30), as indicated by measuring the activity of the heme-dependent catalase. Third, the severe defect in COX activity in Yah1-depleted Gal-YAH1 cells (15) was fully rescued by synthesis of Fdx2, indicating that human Fdx2 can participate not only in yeast mitochondrial Fe/S cluster synthesis but also replace Yah1 function in the hydroxylation of heme O to heme A. Finally, we tested whether expression of Fdx2 can normalize the activation of the yeast iron regulon observed upon functional defects of the mitochondrial iron-sulfur cluster (ISC) assembly and export machineries (31, 32). As a reporter we used the promoter of *FET3*, which encodes the multi-copper ferroxidase of the high affinity iron uptake system (33), fused to GFP. Green fluorescence was strongly increased in Yah1-depleted Gal-YAH1 cells carrying the FET3-GFP construct (Fig. 3*C*) (32). Ectopic expression of human Fdx2 in these cells normalized the GFP fluorescence to wild-type levels. For all these four assay systems, no changes of signals measured for Yah1-deficient cells were observed upon expression of Fdx1 (Fig. 3*C*). Taken together, Fdx2, but not Fdx1 can fully replace yeast Yah1 in all tested biochemical parameters, making Fdx2 the functional Yah1 orthologue.

Fdx2 Depletion Adversely Affects Cellular Iron Metabolism in Human Cells. Iron regulatory protein 1 (IRP1) is a [4Fe-4S] protein that resides in the cytosol where it has aconitase activity (see Fig. 2). When cellular Fe/S cluster assembly becomes limiting (e.g., due to low iron availability or upon defects of the biogenesis machineries), IRP1 loses its Fe/S cluster thereby gaining the ability to bind to stem-loop structures of mRNAs encoding several proteins involved in iron homeostasis (9, 34). IRP1 functions together with the related IRP2 that does not bind an Fe/S cluster, yet is degraded under iron-replete conditions. Examples

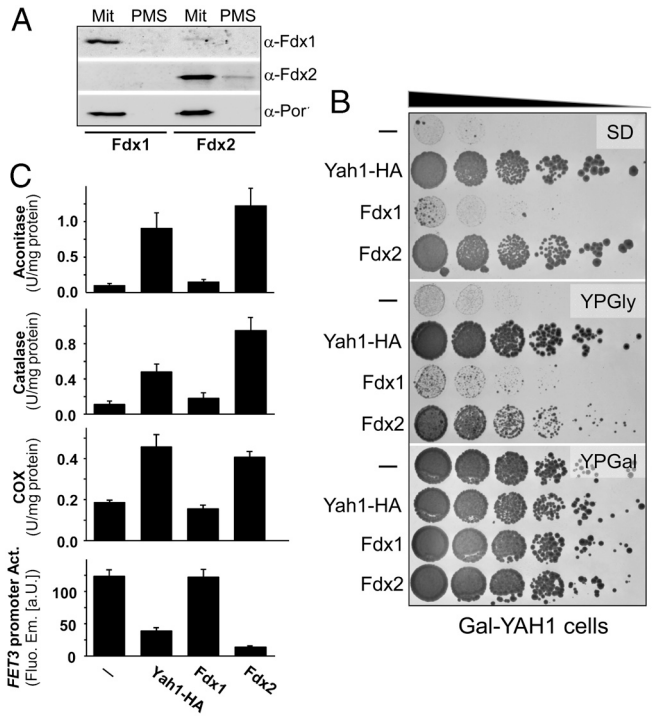

Fig. 3. Fdx2, but not Fdx1, is a functional orthologue of yeast Yah1. Gal-YAH1 yeast cells were transformed with either the empty vector p426-TDH3 (-), p426-YAH1-HA, p426-F1β-Fdx1, or p426-F1β-Fdx2. The latter plasmids encode either mature Fdx1 or Fdx2, N-terminally fused to the mitochondrial presequence of ATPase subunit $F_1\beta$ from *Neurospora crassa* under the control of the *TDH3* promoter. Cells were cultivated for 40 h in synthetic complete minimal medium supplemented with glucose (SD) to deplete Gal-YAH1 cells for Yah1. (*A*) Mitochondria (Mit) and postmitochondrial supernatants (PMS) were isolated and analyzed for Fdx1 and Fdx2 by immunostaining. Porin (Por1) served as a mitochondrial marker. (*B*) Serial dilutions of yeast cells were spotted onto agar plates containing SD medium, or rich YP medium supplemented with either glycerol (YPGly) or galactose (YPGal) and incubated for 3 days at 30 °C. (*C*) Activities of aconitase, catalase and cytochrome oxidase (COX) were determined in extracts prepared from cells described in part B. Cells of part B were transformed with the reporter construct p414-FET3-GFP that encodes GFP under the control of the promoter of *FET3*, an iron-responsive gene. *FET3* promoter activities were assessed by determining the GFP-specific fluorescence of cells after cultivation in SD medium for 40 h (lower right). Error bars represent standard deviations.

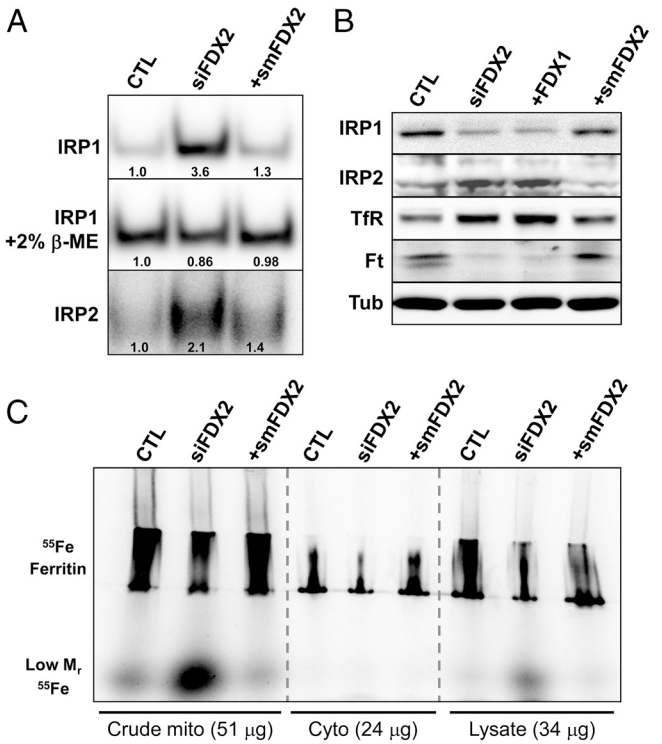

Fig. 4. Fdx2 depletion affects cellular iron metabolism. (*A*) Lysates from HeLa cells transfected as in Fig. 2*C* were analyzed for IRP binding activities by electrophoretic mobility shift assay. Because human IRP1 and IRP2 have the same mobility, anti-IRP1 or anti-IRP2 antibodies were used to supershift the respective proteins (the top panel shows IRP1 activity, with IRP2 supershifted out of the displayed image). Lysates were treated with 2% β-mercaptoethanol (β-ME) to divulge total IRP1 protein levels. Values indicate densitometric evaluation of the bands. (*B*) Total lysates from cells transfected as in Fig. 2*C* were subjected to immunoblotting using the indicated antibodies. TfR, transferrin receptor; Ft, ferritin; Tub, tubulin. (*C*) Cells were transfected as in Fig. 2*C*, treated with 0.5 μM ^{55}Fe-transferrin after the third transfection, and fractionated. Cytosol (Cyto) crude mitochondria (Crude mito) and total lysates (Lysate) were subjected to native-PAGE to resolve iron-containing proteins from low-molecular mass (M_r) iron compounds, and analyzed by autoradiography.

of IRP-regulated mRNAs are those of transferrin receptor (TfR), which is stabilized by IRP binding, and ferritin (Ft), whose translation is inhibited by IRP binding. Because Ft is a ubiquitous iron storage protein and TfR is the gateway of iron entry into most cells, the reciprocal regulation of these two proteins by iron or Fe/S protein biogenesis endows cells with the ability to rapidly adjust iron availability to meet cellular needs. In an RNA electrophoretic mobility shift assay, Fdx2-depleted HeLa cells exhibited a robust increase in IRP1 RNA binding activity compared to controls or cells cotransfected with psmFDX2 (Fig. 4*A*). IRP2 also showed increased mRNA binding activity. Surprisingly, the changes in the protein levels of the two IRPs were opposite under Fdx2 depletion (Fig. 4*B*). For IRP1 the decrease is readily explained by the common observation that apo-IRP1 lacking its Fe/S cluster is more prone to degradation (26, 35). Because IRP2 protein levels are negatively regulated by the iron availability in the cytosol (34), our results suggest that cytosolic iron was diminished. To verify this, we radiolabeled Fdx2-depleted HeLa cells with ^{55}Fe-transferrin for 3 d, performed cell fractionation, and evaluated the distribution of ^{55}Fe by native PAGE. A low molecular mass iron species (36, 37) was dramatically increased in the crude mitochondrial fraction of cells deficient in Fdx2, suggesting mitochondrial iron accumulation when Fdx2 is depleted and hence when Fe/S protein biogenesis is hindered (Fig. 4*C*). The low molecular mass iron species was not detectable in the cytosolic fraction, yet the amount of iron stored in ferritin was decreased. We conclude from these data that depletion of mitochondrial Fdx2 has a severe effect on cellular iron homeostasis, which is most likely initiated by an assembly defect of the Fe/S cluster of IRP1.

Fdx2 Cannot Substitute for Fdx1 in Steroidogenesis. Our results so far indicate that Fdx2 performs a specific function in cellular Fe/S protein assembly. Because this protein is highly homologous to Fdx1, we examined whether it can also participate in the reduction of mitochondrial CYP for steroidogenesis. An electron transfer system was set up in vitro, using NADPH as an electron source, recombinant purified FdxR, Fdx1, Fdx2, and 11beta-hydroxylase (CYP11B1), and 11-deoxycortisol as the substrate (Fig. 5*A*). Using HPLC, we observed an efficient conversion of 11-deoxycortisol into cortisol by CYP11B1 that was dependent on the Fdx1 concentration (Fig. 5*B*, *Upper*). Surprisingly, Fdx2 was highly inefficient in this reaction; even at 20 μM Fdx2 barely any product formation was detected (*Lower*). Quantitative analysis revealed a 500-fold lower rate of conversion by Fdx2 compared to Fdx1 (*Inset*). UV-Vis spectrophotometry confirmed that NADPH/FdxR almost instantly reduced the [2Fe-2S] cluster of Fdx2, indicating that this part of the electron transfer chain does not throttle back the CYP11B1 activity (compare to Fig. 1*B*).

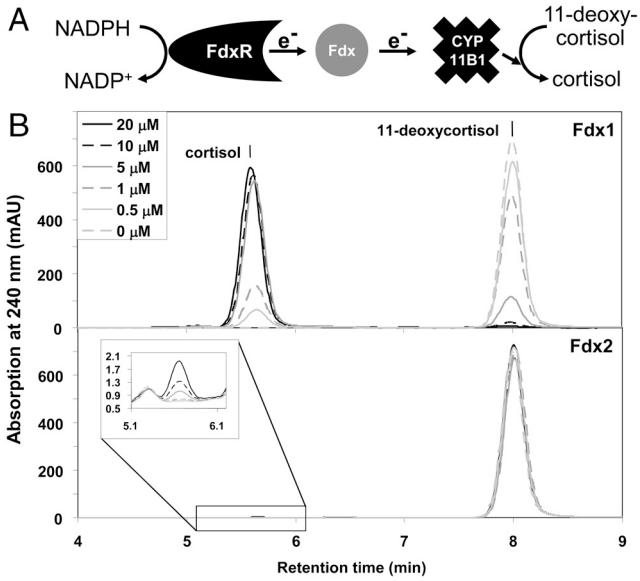

Fig. 5. Fdx1, but not Fdx2, efficiently transfers electrons for steroidogenesis. (*A*) A schematic diagram illustrating the electron transfer from NADPH to 11-deoxycortisol catalyzed by FdxR, Fdx1, and CYP. (*B*) The indicated concentrations of recombinant Fdx1 (*Upper*) or Fdx2 (*Lower*) were incubated for 30 min with recombinant FdxR, mitochondrial cytochrome P450 (CYP11B1), 11-deoxycortisol, and a NADPH-regenerating system. The steroid compounds were organically extracted and analyzed by HPLC. The inset in the lower panel depicts a magnification of cortisol formation by Fdx2.

Taken together, our findings show a high specificity of Fdx1 for steroidogenesis and of Fdx2 for heme A and Fe/S cluster biosynthesis.

Discussion

Our study demonstrates that humans possess two functionally independent mitochondrial [2Fe-2S] ferredoxins. Despite their high sequence similarity these proteins assume highly specific roles in distinct biochemical pathways (Fig. 6). Adrenodoxin (Fdx1) can only reduce mitochondrial CYP enzymes that are essential in adrenal steroidogenesis, bile acid formation, and vitamin D synthesis (6). The newly characterized Fdx2 specifically participates in both heme A and Fe/S protein biosynthesis, as shown by RNAi depletion in human cells and by yeast complementation experiments, which thus establish Fdx2 as the functional orthologue of Yah1. A phylogenetic analysis has defined two subgroups of mitochondrial ferredoxins with Fdx1/Yah1 in one and Fdx2 in the other subgroup (20). Our investigation demonstrates that this subgroup distinction is not reflected in the function of the subgroup members. It will therefore be interesting to unravel what structural features determine the striking functional specificity of Fdx1 and Fdx2. The 3D structure of mammalian Fdx1 has already been resolved (38) and the high level of sequence similarity between Fdx1 and Fdx2 predicts similar structures. Comparative analysis of the primary structures of Fdx1 and Fdx2/Yah1 and the eventual determination of the 3D structure of Fdx2 will make it possible to understand what bestows these ferredoxins with the capacity to interact with specific substrates only. Future study is also needed to determine whether Fdx2 may participate in coenzyme Q synthesis, as has recently been shown for yeast Yah1 (39).

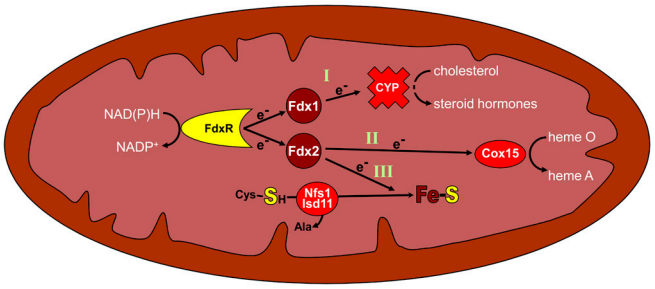

Fig. 6. The two mitochondrial [2Fe-2S] ferredoxins fulfill three distinct roles in mammalian cells. Fdx1 (adrenodoxin) specifically catalyzes reactions central to steroidogenesis (I), whereas Fdx2 is involved in heme A formation and Fe/S protein biogenesis (II and III).

The participation of Fdx2 in two fundamental biosynthetic processes explains well why this protein is present in virtually all tissues. It is surprising that, even in excess, the highly similar Fdx1 cannot take over these functions in human or yeast cells. Conversely, our biochemical studies show that Fdx2 is unlikely to participate in mammalian steroidogenesis. Hence, the two ferredoxins serve as specificity devices for electron transfer from a single donor, the ferredoxin reductase (FdxR), to several different target proteins. Whereas the electron-receiving partners of Fdx1 are six well-known mitochondrial CYPs, the details of the electron transfer reaction for Fdx2/Yah1 are only partially understood to date. Fdx2/Yah1 may directly interact with Cox15 for the conversion of heme O to heme A, even though this interaction remains to be verified (15). Also, it has been speculated that a Fdx-mediated electron transfer might be needed for conversion of the sulfur of cysteine (formally S^0) to the sulfide (S^{2-}) present in Fe/S clusters. This idea is consistent with the fact that Yah1 was shown to act early in Fe/S protein biosynthesis; i.e., for synthesis of a nascent Fe/S cluster on the scaffold protein Isu1 (10).

Unlike in humans, *Saccharomyces cerevisiae* FC does not contain an Fe/S cluster, though blockage in Fe/S protein biogenesis in both species results in decreased heme production (30, 40). Given the intimate connection between the two major iron-utilizing pathways, heme and Fe/S cluster biosynthesis, it is not surprising that cellular iron metabolism is directly regulated by the cell's capacity to generate Fe/S clusters (40). In humans, IRP1 is a major coupler of these two processes, because the [4Fe-4S] cluster status of this protein dictates its ability to control cellular iron homeostasis. Consistent with our finding that Fdx2 is required for Fe/S cluster assembly on IRP1 we report disturbed iron distribution in Fdx2-depleted cells. This is reflected by elevated TfR levels, suggesting an increase in cellular iron uptake, and by a concomitant drop in Ft levels (9, 23). However, the fate of this excess cellular iron that cannot efficiently be stored in Ft has remained unknown. A similar scenario arises in patients with Friedreich's ataxia, in whom a deficiency in mitochondrial frataxin results in decreased Fe/S proteins (41). In these patients, as well as in mouse models of the disease (42), it has been suggested that iron is accumulating in mitochondria. In yeast, the massive mitochondrial iron accumulation under compromised Fe/S biogenesis is well established (40). Our study directly demonstrates mitochondrial accumulation of a low molecular mass form of iron in an acute model of defective human Fe/S protein biogenesis (36).

In summary, humans depend on two functionally noninterchangeable mitochondrial ferredoxins, which participate in fundamental physiological processes. Conspicuously, the newly characterized Fdx2 not only transfers electrons for the biogenesis of mitochondrial heme A and Fe/S clusters, but is also essential for the maturation of cytosolic Fe/S proteins, underscoring the importance of mitochondria in all Fe/S protein production in the eukaryotic cell.

Materials and Methods

Reagents and Cell Lines. Antibodies against Fdx1 and Fdx2 were raised in rabbits using purified proteins produced in *E. coli*. The remaining antibodies were against: mitochondrial aconitase (L. Szweda), GPAT (H. Puccio), $F_1\alpha/\beta$ (H. Schägger), TfR (Zymed), Ft (ICN), tubulin (clone DM1α, Sigma), SDH, and NDUFS3 (MitoScience). Small interfering RNAs (siRNAs) with predesigned sequences were purchased from Applied Biosciences. For all RNAi assays

performed, transfection with two separate scrambled siRNAs yielded identical results as mock transfections. Human tissue lysates were purchased from Axxora, cell culture reagents from PAA Laboratories, and all other reagents from Carl Roth GmbH, unless otherwise noted.

HeLa cells were maintained in DMEM containing 4.5 g/liter glucose and supplemented with 7.5% FCS, 1 mM glutamine, and 1% penicillin-streptomycin. The previously described Gal-YAH1 yeast strain was generated in a W303-1A (*MATα*, *ura3-1*, *ade2-1*, *trp1-1*, *his3-11,15*, *leu2-3,112*) background by exchanging the *YAH1* promoter with a galactose-inducible *GAL1-10* promoter (12).

Plasmids. All oligonucleotides used, including siRNA sequences, are listed in Table S1. Human Fdx1 and Fdx2 cDNA clones in pCMV-SPORT6 were acquired from imaGenes. Using these clones, we performed PCR amplification to clone the genes lacking their putative mitochondrial targeting sequences into pETDuet-1 (Novagen), which was then transformed into BL21 *E. coli* cells for recombinant ferredoxin production. This construct incorporates an N-terminal 6xHis tag, which was used for protein purification on a Ni-nitrilotriacetic acid (NTA) column (GE Healthcare Life Sciences). Additional plasmids were generated by cloning the entire coding regions of the respective genes into pEGFP-N3 (Clontech), thus generating constructs that encode Fdx1 (plasmid pFDX1) or Fdx2 (pFDX2) fused to a C-terminal EGFP. For complementation experiments, pFDX2 was mutated in the coding regions targeted by the siRNAs, using previously described methods (43) to introduce numerous silent mutations (psmFDX2).

Miscellaneous Procedures. Previously described methods were used for harvesting and fractionating HeLa cells with digitonin, transfection by electroporation; measurements of the catalytic activities of the aconitases, complex I, SDH, cytochrome oxidase, CS and lactate dehydrogenase (LDH), yeast aconitase, and catalase (27, 44), estimation of yeast iron regulon activation by *FET3-GFP* (30), in vitro hydroxylation of 11-deoxycortisol to cortisol (19), determination of IRP binding activities (23), native PAGE separation of iron-bound moieties (36), and iron incorporation into respiratory complexes (24, 27). All bar graphs represent the average of at least three independent sets of transfections.

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