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Frataxin deficiency disrupts mitochondrial respiration and pulmonary endothelial cell function

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Abstract

Deficiency of iron-sulfur (Fe-S) clusters promotes metabolic rewiring of the endothelium and the development of pulmonary hypertension (PH) *in vivo*. Joining a growing number of Fe-S biogenesis proteins critical to pulmonary endothelial function, recent data highlighted that

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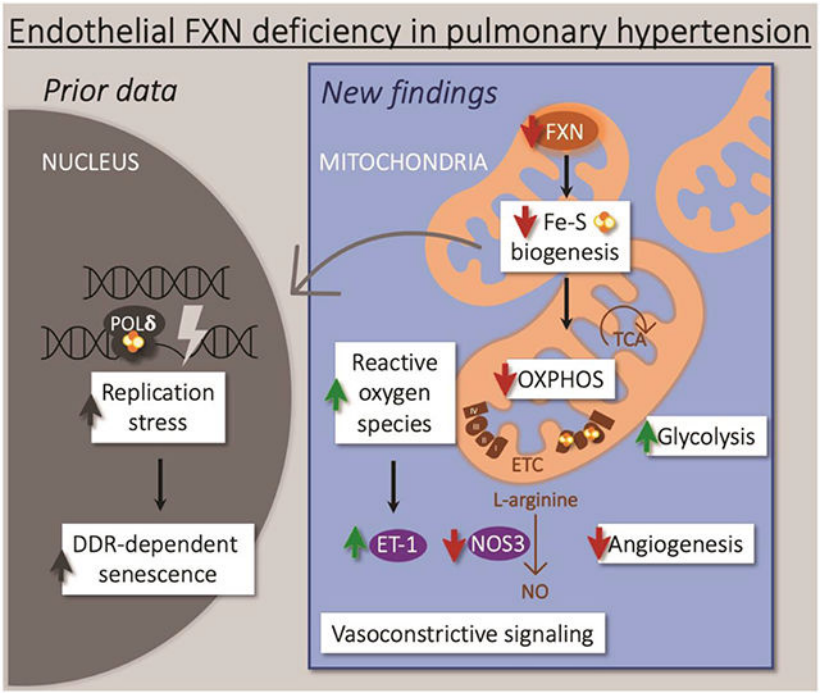
Disclosures

S.Y.C. has served as a consultant for Acceleron Pharma and United Therapeutics. S.Y.C. has held research grants from Actelion, Bayer, and Pfizer. S.Y.C. is a director, officer, and shareholder of Synhale Therapeutics. S.Y.C. and T.B. hold patents and has filed patent applications regarding the therapeutic targeting of metabolism and senescence in pulmonary hypertension.

frataxin (FXN) reduction drives Fe-S-dependent genotoxic stress and senescence across multiple types of pulmonary vascular disease. Trinucleotide repeat mutations in the *FXN* gene cause Friedreich's ataxia, a disease characterized by cardiomyopathy and neurodegeneration. These tissue-specific phenotypes have historically been attributed to mitochondrial reprogramming and oxidative stress. Whether FXN coordinates both nuclear and mitochondrial processes in the endothelium is unknown. Here, we aim to identify the mitochondria-specific effects of FXN deficiency in the endothelium that predispose to pulmonary hypertension. Our data highlight an Fe-S-driven metabolic shift separate from previously described replication stress whereby FXN knockdown diminished mitochondrial respiration and increased glycolysis and oxidative species production. In turn, FXN-deficient endothelial cells had increased vasoconstrictor production (EDN1) and decreased nitric oxide synthase expression (NOS3). These data were observed in primary pulmonary endothelial cells after pharmacologic inhibition of FXN, mice carrying a genetic endothelial deletion of FXN, and inducible pluripotent stem cell-derived endothelial cells from patients with FXN mutations. Altogether, this study indicates FXN is an upstream driver of pathologic aberrations in metabolism and genomic stability. Moreover, our study highlights FXN-specific vasoconstriction *in vivo*, prompting future studies to investigate available and novel PH therapies in contexts of FXN deficiency.

Graphical Abstract



Keywords

frataxin; iron-sulfur; mitochondria; endothelium; pulmonary hypertension

Introduction

Pulmonary hypertension (PH) is a progressive and fatal disease of the lung vasculature, driven in part by pathologic changes in pulmonary artery endothelial cells. Specifically, imbalance in the production of vasoactive mediators, such as increased vasoconstrictor endothelin-1 (EDN1) and decreased nitric oxide (NO) via downregulation of nitric oxide synthase (NOS3) as well as dysregulated angiogenesis ultimately contribute to pulmonary vascular disease [1-3].

Recently, we demonstrated that endothelial deficiency of frataxin (FXN), a nuclear-encoded, mitochondrial Fe-S biogenesis gene [4, 5], predisposed to PH development *in vivo* [6]. Specifically, both genetic and acquired FXN deficiency promoted Fe-S-dependent replication stress, DNA damage, and acute growth phase arrest, leading to subsequent endothelial senescence. This work supports the notion that PH may represent a previously underappreciated clinical phenotype in patients with Friedreich's ataxia (FRDA) – a disease driven by trinucleotide repeat mutations in the *FXN* gene and defined by severe neurodegeneration and cardiomyopathy – as we and others have demonstrated robust pulmonary vascular remodeling in FRDA patients [7, 8]. Importantly, hypoxia, via a HIF-dependent pathway, promoted a FXN-deficient subpopulation of endothelial cells. Hypoxia also downregulated FXN in other vascular cell types [6] but not all tissues in general [9, 10], indicating a level of vascular cell-type specific regulation. Additionally, while the nuclear genotoxic effects of pulmonary endothelial FXN reduction have been studied [6], data in other cell types, specifically those with high bioenergetic capacity such as the nervous system, myocardium, and endocrine pancreas, have highlighted how FRDA-dependent FXN deficiency also drives mitochondrial dysfunction [5].

Mitochondrial dysfunction is a well-accepted driver of pulmonary vascular disease and of endothelial dysfunction specifically [11]. A hallmark of the metabolic reprogramming observed in the endothelium in PH is the shift from oxidative phosphorylation to glycolysis [12-15], similar to the Warburg effect in cancer [16, 17]. Iron-sulfur (Fe-S) clusters are essential, redox-capable cofactors [18], that allow for effective respiration [19]. Of note, we have previously shown that deficiency of Fe-S cluster biogenesis proteins, including ISCU1/2 and BOLA3, contribute to the disruption of endothelial cell metabolism and PH *in vivo* [1-3]. Loss of ISCU1/2 and BOLA3 resulted in endothelial metabolic reprogramming in the form of the reduced respiration with a concomitant increase in aerobic glycolysis [1-3], reactive oxygen species (ROS) formation [1, 3], and lipoate-dependent regulation of glycine in PH [3]. These Fe-S-specific metabolic changes promoted pathological changes in pulmonary artery endothelial cells, consistent with PH. However, it is not known whether deficiency of FXN controls endothelial metabolic and consequent pathophenotypic reprogramming.

Materials and Methods

Animal model

As described [6], to generate endothelial-specific knockout of FXN, FXN flox/flox (FXN f/f) mice were crossed with Cdh5(PAC)-ERT2+-Cre (EC FXN −/−) recombinase. Mice were

administered (30 mg/kg) tamoxifen for three days consecutively at 10 weeks. After two weeks, mice were subjected to hypoxia (10% O2) for the next three weeks. Lung tissue was harvested for analysis.

Statistics

All data represent three independent experiments with at least n=3 technical replicates and are presented as mean +/− SD unless otherwise specified. Paired samples were compared by a two-tailed Student's *t*-test for normally distributed data and Mann-Whitney U non-parametric testing for non-normally distributed data. For comparison among greater than two groups, oneway or two-way ANOVA with post-hoc Tukey's analysis to adjust for multiple comparisons was performed. Significance was defined by a p-value less than 0.05.

Study Approval

All experiments involving animals were approved by the University of Pittsburgh (DLAR). Experiments involving the use of human tissue were approved by the University of Pittsburgh IRB. Informed consent was obtained for research use of collected tissue. Ethical approval and informed consent for all procedures in this study conformed to standards of the Declaration of Helsinki.

Additional Information

Additional methods are found in Supplementary Material.

Results

Acquired FXN deficiency attenuates Fe-S-dependent mitochondrial metabolism.

To directly assess mitochondrial respiration, Seahorse extracellular flux analysis was utilized to measure glycolysis (extracellular acidification rate (ECAR)) and oxidative phosphorylation (a component of whole-cell oxygen consumption rate (OCR)) in FXN-deficient PAECs (Supplemental Figure 1A). At baseline and following termination of oxidative phosphorylation by the ATP synthase inhibitor oligomycin (indicative of glycolytic capacity), FXN knockdown increased ECAR (Figure 1A-C) and expression of glycolytic markers (Supplemental Figure 1B**)** but did not alter OCR (Figure 1D-E). Under high glucose conditions, sustained OCR was not dependent upon fatty acid oxidation, as evidenced by a negligible change in baseline OCR with etomoxir, an inhibitor of CPT1a and fatty acid oxidation **(**Supplemental Figure 1C**).** Instead, by reducing the available glucose, and thus flux through the mitochondria, we found that FXN deficiency decreased baseline and ATP-linked respiration (Figure 1F-H), consistent with the notion that oxidative phosphorylation is attenuated due to loss of Fe-S centers in the electron transport chain. Measures of maximal respiratory capacity, proton leak, and non-mitochondrial oxygen consumption were not significantly different between groups **(**Supplemental Figure 1D and E**).** FXN did not control Fe-S-dependent synthesis of lipoic acid and thus did not affect the glycine cleavage system **(**Supplemental Figure 1F**).**

Corresponding with this metabolic rewiring, FXN deficiency in PAECs increased reactive oxygen species (ROS), as measured by intracellular hydrogen peroxide levels (Figure 1i-J).

Thus, knockdown of FXN promotes metabolic rewiring and an imbalance in mitochondrial ROS in PAECs. To determine whether mitochondrial oxidative stress directly promotes DNA damage previously demonstrated in endothelial cells with FXN deficiency [6], we employed MnTBAP, a cell-permeable mimetic of superoxide dismutase and peroxynitrite scavenger, to reduce mitochondria-derived ROS. Using an Amplex red assay, MnTBAP treatment prevented the increase in hydrogen peroxide dependent upon FXN knockdown **(**Supplemental Figure 2A**).** Next, markers of replication stress (p-RPA32/RPA32), the DNA damage response (p-ATR/ATR, CHK1, Ub-yH2AX/yH2AX), and growth arrest ($p21^{Cip}$) were then measured by immunoblot under similar treatment conditions. Notably, FXN-deficient elevation of these genotoxic stress markers was not prevented by abrogation of mitochondrial ROS production **(**Supplemental Figure 2B**),** supporting simultaneous but separate FXN-driven mitochondrial and nuclear dysfunction.

FXN deficiency induces pulmonary artery endothelial dysfunction.

Accompanying alterations in mitochondrial metabolism, we found that FXN deficiency in PAECs promotes pathologic changes in endothelial vasomotor function, migration and angiogenesis, in addition to DDR-dependent senescence previously described [6]. Namely, in PAECs both transcript and secreted protein levels of the vasoconstrictive mediator endothelin-1 (*EDN1*/ET-1) were increased with FXN knockdown (Figure 2A-B). Moreover, the combination of hypoxia and FXN knockdown exacerbates the phenotype (Supplemental Figure 3A-B). Demonstrating the role of oxidative stress in this response, EDN1 up-regulation was abrogated by MnTBAP treatment (Figure 2C). When cultured in collagen gel, primary pulmonary artery smooth muscle cells (PASMCs) exhibited increased contraction after exposure to media from FXN-deficient hypoxic PAECs, which could be reversed with the ET-1 receptor antagonist ambrisentan (Supplemental Figure 3C). FXN knockdown also reduced mRNA and protein expression of nitric oxide synthase 3 (NOS3), the enzyme required for production of the vasodilator nitric oxide (NO) (Figure 2D-E).

Like EDN1, NOS3 down-regulation was more pronounced during FXN knockdown in combination with chronic hypoxia (Supplemental Figure 3D-E). Importantly, total nitrite and nitrate levels, a surrogate measurement for NO, were diminished (Figure 2F), indicating a decrease of NO production by FXN-deficient PAECs and thus enhancing the vasoconstrictive phenotype. Under hypoxic conditions when endogenous FXN was downregulated in endothelial cells [6], forced expression of FXN in hypoxic conditions (Supplemental Figure 3F) increased NOS3 (Supplemental Figure 3G), demonstrating that FXN is both necessary and sufficient for NOS3 expression.

Finally, FXN knockdown, in normoxic and hypoxic conditions, decreased endothelial cell migration during a scratch assay (Figure 2G, Supplemental Figure 3H) as well as decreased branch points (Figure 2H, Supplemental Figure 3I) and total tube length (Figure 2I, Supplemental Figure 3J), consistent with reduced angiogenic potential. Since FXN knockdown does not affect endothelial viability [6], the observed decrease in migratory and angiogenic capacity did not result from changes in PAEC survival. In sum, FXN deficiency in PAECs leads to a dysfunctional endothelium characterized by metabolic stress, an imbalance in vasomotor tone mediators, and diminished angiogenic potential.

Genetic FXN deficiency mirrors the metabolic dysfunction that drives endothelial dysfunction.

To determine whether genetic FXN deficiency produces similar endothelial metabolic dysfunction, inducible pluripotent stem cells (iPSCs) from both male and female patients with FRDA mutations were differentiated into endothelial cells (iPSC-ECs) [20] and compared to gender- and age-matched controls without FXN mutations. FRDA iPSC-ECs exhibited markedly reduced FXN levels (Supplemental Figure 4A). To confirm endothelial differentiation, iPSC-ECs were evaluated for expression of endothelial markers and assayed for angiogenic potential. FRDA iPSC-ECs exhibited comparable endothelial cell expression markers (Supplemental Figure 4B-C). FRDA iPSC-EC cell lines demonstrating obvious angiogenic phenotype were selected for experimentation. Since comparative assays for angiogenic potential would be inherently confounded, we focused on evaluating mitochondrial dysfunction and mediators of vasomotor tone in FRDA iPSC-ECs. Similar to acquired FXN deficiency, iPSC-ECs with FXN mutations showed an increase in intracellular hydrogen peroxide (Figure 3A, Supplemental Figure 4D). This FXN-driven oxidative stress is accompanied by elevated endothelin-1 transcript and secreted protein levels (Figure 3B-C, Supplemental Figure 4E-F) as well as reduced NOS3 transcript levels (Figure 3D, Supplemental Figure 4G), signifying pathogenic alteration in vasomotor tone effectors similar to those in FXN-deficient primary PAECs. However, overexpression of FXN did not reverse NOS3 downregulation or EDN1 upregulation in chronically and genetically FXN deficient FRDA iPSC-ECs (Figure 4H-J).

Endothelial FXN deficiency promotes dysregulation of endothelial vasomotor mediators in vivo.

We sought to study FXN deficiency in the pulmonary endothelium *in vivo*. To do so, tamoxifen-dependent endothelial (EC) *Fxn−/−* mice (Cdh5(PAC)-Cre-ERT2+ mice crossed with Fxn *flox/flox* mice were exposed to normoxia vs. chronic hypoxia to induce PH [6]. As previously described, such endothelial-specific FXN deletion in chronic hypoxia drove more severe hemodynamic and histologic manifestations of PH [6]. Importantly, consistent with cultured PAECs and iPSC-ECs harboring FXN deficiencies (Figure 2), endothelial FXN-deficient mice demonstrated decreased NOS3 (Figure 4A) and increased EDN1 levels (Figure 4B). Thus, loss of endothelial FXN results in the imbalance in pulmonary vasomotor tone mediators *in vitro* and *in vivo* – a process that contributes, at least in part, to the overall manifestations of PH.

Discussion

Endothelial mitochondrial dysfunction [11] and DNA damage [21-25] have been separately linked to PH, but any shared mechanistic regulation of these dynamic endothelial phenotypes was previously undefined. Building upon data that links FXN reduction to Fe-S-specific nuclear damage [6], FXN deficiency disrupted mitochondrial function by preventing glucose oxidation which led to upregulated glycolytic activity and reactive oxygen species. Similar to other Fe-S biogenesis genes [1-3], this mitochondrial oxidative stress resulted in increased EDN1 and decreased NOS3 expression as well as reduced migratory and angiogenic capacity. Our data now cumulatively support that FXN controls

not only genotoxic but also metabolic stress resulting in pathophenotypes beyond senescent cell fate (Graphical Abstract). For the first time, we demonstrate FXN-dependent endothelial metabolic dysfunction across cellular, animal, and human models of PH.

While the shift from oxidative phosphorylation to aerobic glycolysis has been observed in patient and model tissues with FRDA [5], to our knowledge, our data now demonstrate consistent changes in the endothelial cell (Figure 1). Whether additional metabolic pathways (*e.g.*, pentose phosphate pathway (PPP)) are dysregulated in FXN-deficient endothelial cells is not yet known. While these pathways do not include Fe-S-containing proteins, loss of appropriate Fe-S-dependent glucose oxidation alone could lead to compensatory alterations in bioenergetic production. For example, the PPP is often augmented in parallel with glycolysis, and separately, PPP flux is upregulated in pulmonary vascular cells in multiple PH models [14, 26-28]. Moreover, whether FXN deficiency disrupts mitochondrial biogenesis, dynamics, or even mtDNA integrity requires further investigation. Finally, it is important to recognize that precise aspects of this relationship linking hypoxia to FXN deficiency and mitochondrial metabolic dysfunction may be specific to the vasculature. Given that hypoxia upregulates FXN expression in some non-vascular cell types [9, 10] and hypoxia can rescue mitochondrial dysfunction in other tissues [29], our results highlight the complex and context specificity of hypoxic regulation of FXN.

Our data establish that mitochondria-derived oxidative species do not fully account for the genotoxic stress observed in the nucleus (Supplemental Figure 2B). In tandem with our data on disruption of the Fe-S-containing polymerases in FXN-deficient endothelial cells [6], these data support the notion that there may exist two separate organelle-specific processes related to FXN loss. However, this data does not preclude the possibility of bi-directional signaling between organelles, particularly over the course of disease. In particular, FXN-dependent metabolic changes may contribute to the senescent cell fate previously reported in the FXN-deficient endothelium [6]. Mitochondrial dysfunction-associated senescence (MiDAS) represents another stress response pathway like telomere instability or the DNA damage response that converges on permanent growth arrest, but the mechanisms remain incompletely defined [30]. We acknowledge that further experimentation is needed to determine the importance of mitochondrial versus nuclear stress in PH, as these may provide insight into the value of metabolic versus genomic treatment intervention. Moreover, the potential reciprocal relationships between these organelles may favor targeting FXN directly and require the identification of new drugs.

While our data mirror the same endothelial pathophenotypes demonstrated with other Fe-S biogenesis gene deficiencies with Warburg-like metabolic shifts [1-3], only FXN-dependent EDN1 up-regulation could be reversed by targeting mitochondrial superoxide (Figure 2C). Further investigation is needed to define the precise mechanisms that result in changes in vasomotor tone and angiogenesis. In the context of FXN-dependent down-regulation of NOS3 expression (Figure 2D-E), a possible explanation could center on ferrochelatase activity [31], the Fe-S-dependent, rate-limiting enzyme in heme synthesis. Therefore, FXN deficiency may impair heme production and ultimately reduce the expression and activity of NOS3, a heme-containing enzyme [32]. Separately, because nitric oxide can directly bind

and damage Fe-S clusters, endothelial cell NOS3 may be inhibited by another undefined feedback mechanism in conditions of FXN deficiency and reduced Fe-S biogenesis [33].

Interestingly, certain pathophenotypes were not reversible in FRDA-patient derived endothelial cells with overexpression of FXN. Such results are consistent with the finding of senescence exhibited by chronically FXN-deficient cells [6] coupled with existing literature of the irreversible nature of certain phenotypes in senescent cells [34, 35]. Future studies are warranted to determine if such putative irreversibility stems from the chronicity, severity, and/or etiology (i.e., genetic vs. acquired trigger) of FXN deficiency. As such, our data suggest potential challenges of gene therapy alone to treat FRDA pulmonary vasculopathy, which has been observed in autopsy specimens in FRDA patient cells [6].

On the other hand, mice with endothelial FXN deficiency exhibited EDN1 and NOS3. Although further investigations would be required, this suggest current PH-specific vasodilatory therapies that inhibit ET-1 and enhance NO signaling may be effective in circumstances of endothelial FXN deficiency, particularly in FRDA. However, certain types of PH patients do not respond to vasodilatory therapy, including most Group 2 and Group 3 PH patients; yet, FXN deficiency has been identified across Group 1-3 PH subtypes. This may be reflective of the fact that FXN-deficient endothelial cells only represent a subpopulation rather than a predominance of the endothelium in the lung vasculature. In this way, the senescence-dependent inflammatory phenotype may be more important to the pathophysiology of PH as compared to the contribution to vasoconstrictive signaling. Of note, reduced NO availability has been appreciated in senescent endothelial cells [36] and repletion of NO signaling prevented senescence [37]. While the onset of FXN-dependent alterations in vasomotor tone effectors predate the onset of senescence, the vasodilatory therapies may serve a dual purpose that includes preventing endothelial senescence over the course of the disease.

In conclusion, these findings support FXN as a lynchpin connecting Fe-S-related endothelial metabolic reprogramming and genotoxic stress to PH development, reinforcing the importance of Fe-S biology in endothelial cell function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Portions of this manuscript and data were included in M.K.C.'s unpublished doctoral dissertation (Culley, Miranda (2020) *Frataxin deficiency coordinates iron-sulfur dependent metabolic and genomic stress to promote endothelial senescence in pulmonary hypertension*. Doctoral Dissertation, University of Pittsburgh). This study used inducible pluripotent stem cell samples from patients with FRDA (GM23404, GM23913) in the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research. We also thank Y. Lu, S. Annis, and M. Reynolds for their technical assistance.

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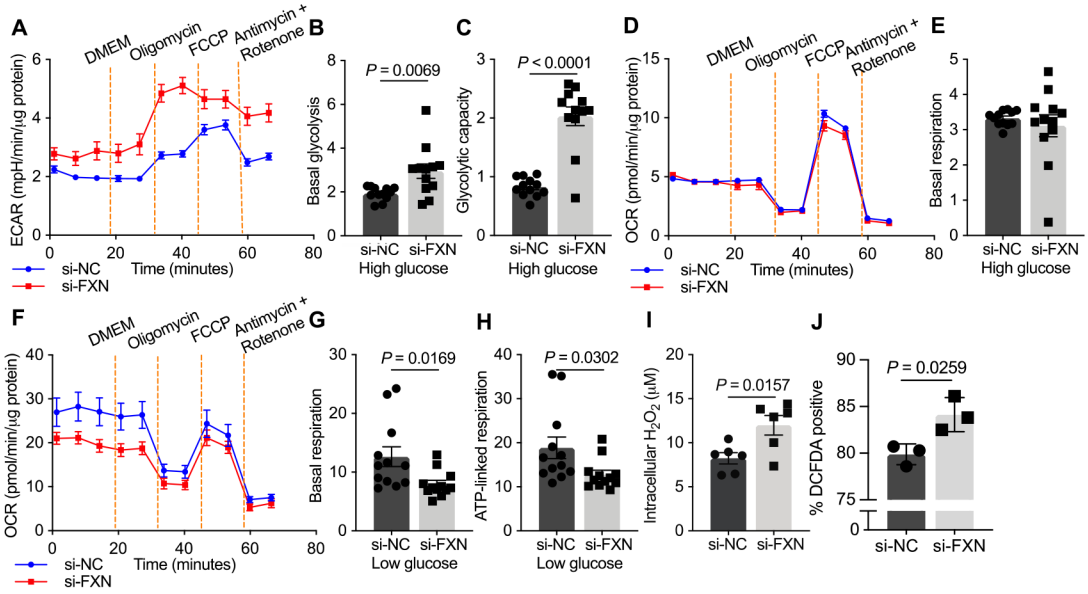

Figure 1. FXN deficiency abrogates mitochondrial respiration and increases oxidative stress.

(**A-H**) Seahorse extracellular flux analysis measurements of PAECs transfected with FXN siRNA (red) or negative control (blue) (n= 12/group) in response to control (DMEM), the ATP synthase inhibitor oligomycin (1μM), the uncoupler FCCP (0.5μM), and the Complex I and Complex III inhibitors rotenone (2μM) and antimycin (0.5μM). Error bars reflect mean +/− SEM. (**A**) Extracellular acidification rate (ECAR) of PAECs cultured in high glucose (25mM). (**B**) Basal glycolysis (post DMEM). (**C**) Glycolytic capacity (post oligomycin). (**D**) Oxygen consumption rate (OCR) of PAECs in high glucose. Error bars reflect mean +/− SEM. (**E**) Basal respiration (post DMEM). (**F**) OCR of PAECs in low glucose (1g/L). (**G**) Basal respiration (post DMEM). (**H**) ATP-linked respiration (post oligomycin). (**I**) Amplex red colorimetric assay measuring intracellular hydrogen peroxide (H_2O_2) in PAECs transfected with FXN siRNA compared to control (n=6/group). (**J**) Flow cytometric percentage of cellular DCFDA fluorescence positivity (n=3/group). Experiments were performed at least three separate times with at least three technical replicates and analyzed by two-tailed Student's *t*-test.

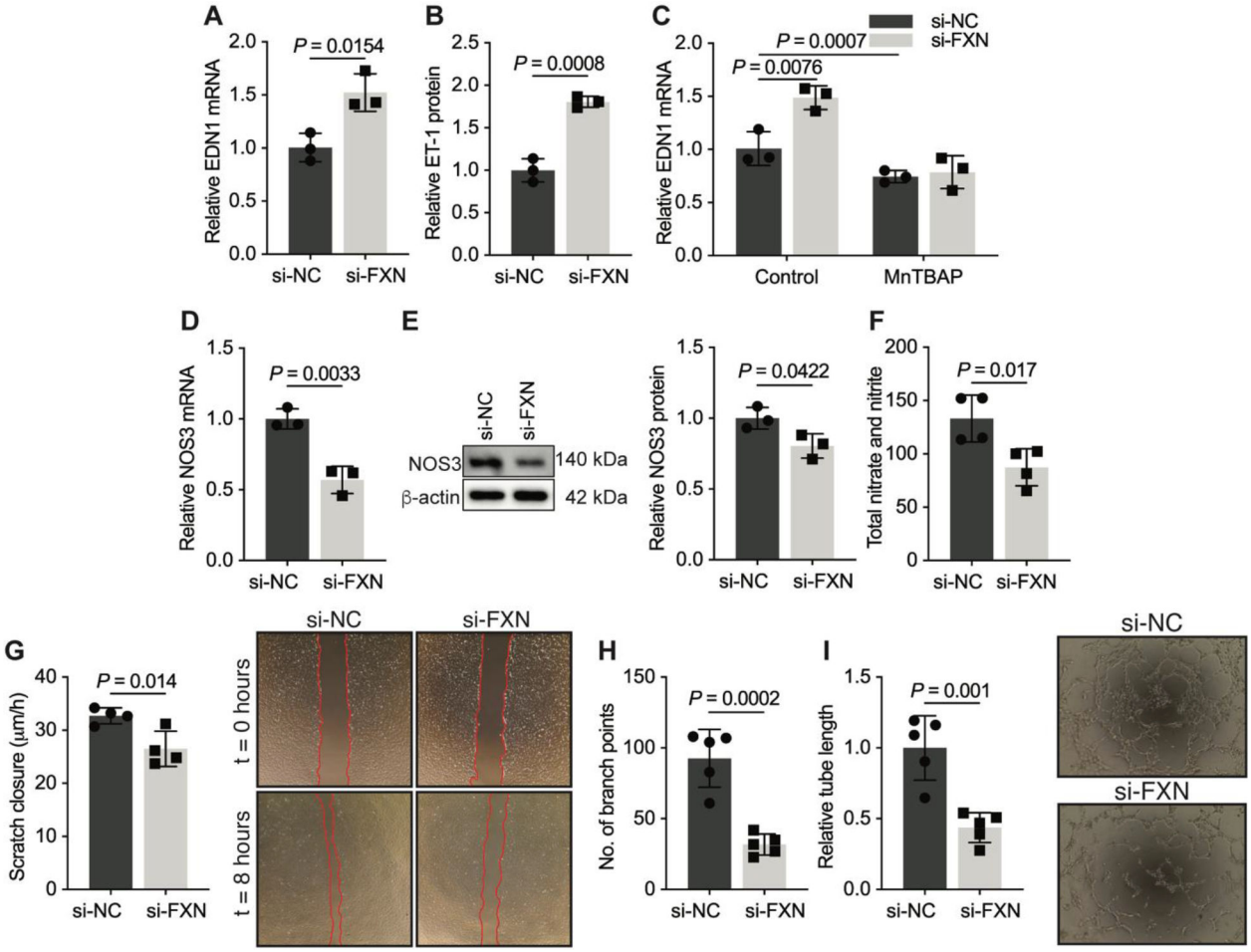

Figure 2. Reduced FXN leads to an imbalance in vasomotor tone effectors and inhibits angiogenesis.

(**A** and **B**) RT-qPCR and ELISA measuring endothelin-1 (*EDN1*/ET-1) expression in PAECs transfected with FXN siRNA or negative control (n=3/group). (**C**) EDN1 mRNA levels in FXN-depleted or FXN-replete PAECs treated with the mitochondrial-specific superoxide dismutase MnTBAP (50μM) or vehicle control (n=3/group). (**D** and **E**) RT-qPCR and immunoblot of endothelial nitric oxide synthase (NOS3) expression (n=3/group). (**F**) Total nitrate and nitrite levels (ng/ng) as measured by Griess reagent colorimetric assay (n=4/group). (**G**) Migration rate over 12 hours of FXN-deficient and control PAECs quantified from a scratch assay with representative images (n=3/group). (**H** and **I**) Number of branch points with representative images and relative tube length reflecting angiogenesis of PAECs cultured in Matrigel (n=4-5/group). Two-tailed Student's *t*-test with error bars that reflect mean +/− SD. Experiments were performed at least three separate times with at least three technical replicates.

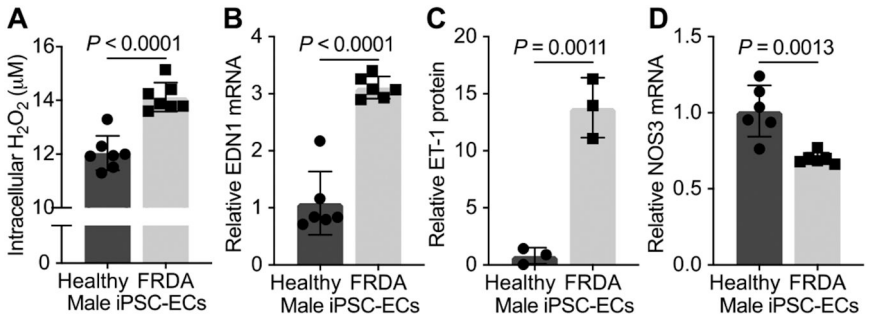

Figure 3. iPS-derived endothelial cells with FXN mutations exhibit similar metabolic and vasomotor pathophenotypes.

(**A-D**) All phenotypic experiments performed in male age-matched inducible pluripotent stem cell-derived endothelial cells (iPSC-ECs) from a patient with FXN mutations (FRDA) compared to control. (**A**) Amplex red colorimetric assay measuring intracellular hydrogen peroxide (H_2O_2) (n=6/group). (**B** and **C**) Relative endothelin-1 transcript (EDN1, n=6/group) and secreted protein (ET-1, n=3/group) expression. (**D**) RT-qPCR of nitric oxide synthase (NOS3) transcript in mutated versus healthy endothelial cells (n=6/group). Two-tailed Student's *t*-test with error bars that reflect mean +/− SD.

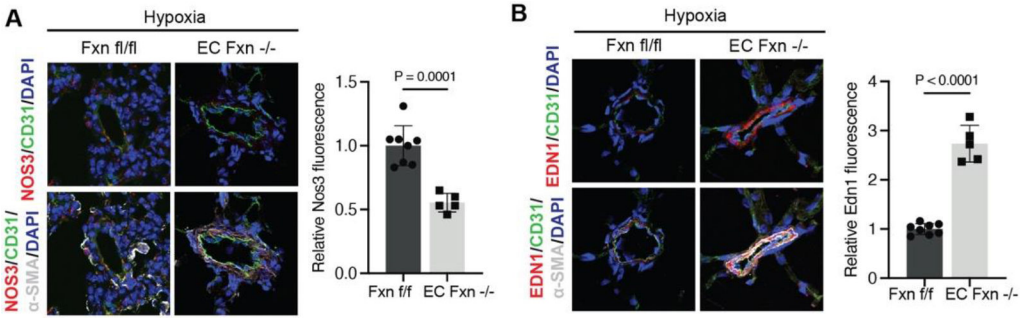

Figure 4. Endothelial FXN deficiency alters the expression of vasomotor mediators in vivo.

(**A**) Confocal microscopic imaging and quantification of pulmonary vascular NOS3 (red), CD31 (green), α-SMA (white), and DAPI (blue) in lung (n=8 Fxn f/f, n=5, EC Fxn −/−). **(B)** Confocal microscopic imaging and quantification of vascular EDN1 (red), CD31 (green), α-SMA (white), and DAPI (blue) in lung (n=8 Fxn f/f, n=5, EC Fxn −/−). Two-tailed Student's *t*-test with error bars that reflect mean +/− SD.