The transcriptional regulator CCCTC-binding factor limits oxidative stress in endothelial cells

Received for publication, August 28, 2017, and in revised form, March 28, 2018. Published, Papers in Press, April 2, 2018, DOI 10.1074/jbc.M117.814699

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Edited by Velia M. Fowler

The CCCTC-binding factor (CTCF) is a versatile transcriptional regulator required for embryogenesis, but its function in vascular development or in diseases with a vascular component is poorly understood. Here, we found that endothelial Ctcf is essential for mouse vascular development and limits accumulation of reactive oxygen species (ROS). Conditional knockout of Ctcf in endothelial progenitors and their descendants affected embryonic growth, and caused lethality at embryonic day 10.5 because of defective yolk sac and placental vascular development. Analysis of global gene expression revealed Frataxin (Fxn), the gene mutated in Friedreich’s ataxia (FRDA), as the most strongly down-regulated gene in Ctcf-deficient placental endothelial cells. Moreover, in vitro reporter assays showed that Ctcf activates the Fxn promoter in endothelial cells. ROS are known to accumulate in the endothelium of FRDA patients. Importantly, Ctcf deficiency induced ROS-mediated DNA damage in endothelial cells in vitro, and in placental endothelium in vivo. Taken together, our findings indicate that Ctcf promotes vascular development and limits oxidative stress in endothelial cells. These results reveal a function for Ctcf in vascular development, and suggest a potential mechanism for endothelial dysfunction in FRDA.

The CCCTC-binding factor (CTCF) is a highly conserved versatile transcriptional regulator that interacts with DNA and multiple protein partners (1–3). CTCF is mainly known for its function as a genomic insulator, and as a mediator of long-range genomic interactions (1, 4–7); however, it can also promote gene expression as a member of transcriptional activation complexes (1, 3). Constitutive Ctcf depletion in mice results in death during early embryogenesis (8). We are only beginning to understand how Ctf controls specific mammalian development processes (7, 9–14). For instance, Ctf interacts with myogenic master regulators to control myogenic cell differentiation and muscle development (2, 3). In addition, Ctf acts as a mediator of long-range genomic interactions to control limb and heart development (6, 9, 14). Overexpression experiments revealed that CTCF limits retinal angiogenesis by preventing enhancer-mediated activation of the gene encoding vascular endothelial growth factor (VEGF) (15). Whether CTCF controls development of the vascular system has not been investigated.

Development of the mouse vasculature begins during gastrulation at embryonic day (E) 6.5 with migration of a subpopulation of mesodermal precursors from the primitive streak toward the embryo proper and extraembryonic tissues, i.e. the yolk sac and placenta (16–19). Development of the vascular network begins with formation of new vessels by vasculogenesis, followed by branching of preexisting vessels by angiogenesis in the yolk sac (16). Blood starts circulating after formation of the primitive vascular plexus in the yolk sac and the embryo proper at E8.25, promoting vascular plexus remodeling into a complex network (16). Vasculogenesis after choioallantoic fusion at E8.0 initiates placental vascular development, and branching angiogenesis forms a complex placental vascular network known as the labyrinth, which mediates nutrient and gas exchange between the mother and the developing embryo (16, 17). Endothelial transcriptional programs coordinate vascular development (18–21). Transcriptional misregulation in endothelial cells in embryonic and extra embryonic vascula-
Ctcf controls vascular development

ture can cause cardiac and vascular defects leading to disease (22, 23).

Friedreich’s ataxia (FRDA) is the most common hereditary neurodegenerative disease (24). Vascular defects and endothelial dysfunction may contribute to FRDA. Impaired vascularization might contribute to muscle fatigability (25). In addition, cardiomyopathy in FRDA is associated with microvascular disease (26). Furthermore, FRDA patients exhibit decreased flow-mediated dilation in the brachial artery, suggesting that endothelial dysfunction may contribute to FRDA (27). FRDA is caused by abnormal expansion of GAA trinucleotide repeats at intron 1 of the Frataxin (FXN) gene that results in decreased protein levels (28), and increased levels of reactive oxygen species (ROS) and oxidative stress (29, 30). FXN is a mitochondrial protein involved in the assembly of iron and sulfur clusters (31) expressed mainly in tissues with high metabolic rates, such as the heart and brown fat (32). GAA trinucleotide repeat expansion triggers silencing of FXN gene expression via epigenetic mechanisms (33). For instance, in FRDA patients’ cells and mouse models, histones located near the expanded GAA repeats are occupied with the repressive mark histone H3 lysine 9 trimethylation (H3K9me3), and have reduced levels of acetylated core histones, which mark transcriptionally active genes (34–36). These modifications might interfere with the activity of transcriptional regulators controlling FXN expression. Studies on fibroblasts and cerebellum from FRDA patients showed that CtCF binding is required to maintain transcriptionally active chromatin, as its depletion from the 5’ untranslated region (5’-UTR) of FXN results in heterochromatin formation. Whether CTCF controls FXN gene expression in endothelial cells, and regulates vascular development is unknown.

Results

Ctcf is expressed in developing and adult mouse vascular endothelium

Ctcf is broadly expressed (3). However, its expression in embryonic or adult vascular endothelial cells has not been investigated. To visualize Ctcf protein in developing vascular endothelium, we performed immunofluorescence for Ctcf and platelet endothelial cell adhesion molecule 1 (Pecam-1), an endothelial marker, on sagittal sections of mice at E9.5, E11.5, postnatal day 2, and 6-week-old adults. Ctcf was detected in nuclei ubiquitously, and was present in vascular endothelial cell nuclei in the third branchial arch, outflow tract, aorta, and pulmonary artery (Fig. 1A). Thus, Ctcf is expressed in vascular endothelial cells throughout embryogenesis and in adulthood.

Ctcf in endothelial progenitors and their derivatives is essential for embryogenesis

Ctcf controls important developmental processes (7, 9–13), but its function in vascular development is unknown. To uncover the function of Ctcf in vascular development, we conditionally inactivated Ctcf in mouse endothelial progenitors and their derivatives by cre-mediated homologous recombination of a floxed allele. Exons 3 to 12 of Ctcf are flanked by LoxP sites in the Ctcf floxed allele (37), which was crossed with Tie2-cre transgenics (38). Efficiency of Ctcf depletion in endothelial cells was evaluated by immunofluorescence for Ctcf and Pecam-1. Ctcf mutants had over 90% fewer cell nuclei that were double positive for Pecam-1 and Ctcf compared with controls (Fig. S1, A and B).

Embryos with Ctcf-deficient endothelial progenitors and derivatives died by E11.5 (Fig. S1C). Ctcf mutant embryos at E9.5 had no gross morphological defects. E10.5 embryos were smaller (Fig. 1B), suggesting deficient growth. Mutants developed an overall normal heart, with normal ventricular wall
thickness, but appeared to have reduced trabeculae at E10.5 (Fig. S1, D and E). To determine whether deficiency of Ctf
affects embryonic vasculature patterning we stained Pecam-1 in whole control and Ctf mutant embryos. Ctf mutants had an overall normal vasculature pattern (Fig. S1F). Accordingly, the number of major branches of the cerebral vasculature was comparable between control and mutant embryos at E9.5 and E10.5 (Fig. 1, C and D). In contrast, quantification of cerebral vessel diameter revealed narrower vessels in Ctf mutants than controls at E9.5 and E10.5 (Fig. 1E). Thus, endothelial Ctf is required for embryogenesis and might regulate vascular development.

**Ctf is required for yolk sac vascular remodeling**

The developing vascular network extends into the yolk sac and placenta (16). Defects in the yolk sac vasculature can compromise embryonic development (20, 39, 40). Defective yolk sac vasculature might affect embryogenesis in Ctf mutants. To test this possibility, we analyzed the vascular network in the yolk sac of Ctf mutant and control embryos at E8.5, E9.5, and E10.5. Ctf mutant yolk sacs at E8.5 had comparable vessel area, vessel length and lacunarity, a measure of the average gap between blood vessels and reflective of vessel disorganization (41) (Fig. 2, B and C). In contrast, E9.5 mutant yolk sacs had a decreased vessel area and vessel length, and increased lacunarity. Similarly, E10.5 Ctf mutant yolk sacs had decreased vessel area and increased lacunarity (Fig. 2, B and C). These changes were not because of deficient endothelial cell proliferation or increased apoptosis. The number of cells double positive for Pecam-1 and phosphorylated histone H3, were comparable between yolk sacs of control and Ctf mutant embryos at E9.5. Cells double positive for Pecam-1 and activated caspase 3 were absent in control and mutant yolk sacs (Fig. S2).

Blood circulation causes shear stress and induces pressure on blood vessels, stimulating yolk sac vascular remodeling from E8.5 to E9.5 (16, 42). Lack of circulating blood in cultured embryos blocks remodeling of the yolk sac vasculature and causes a dramatic down-regulation of the mechanosensor, endothelial nitric-oxide synthase (eNOS) (encoded by Nos3) (16, 42). Deficient yolk sac vascular remodeling in Ctf mutants

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**Figure 2. Vascular remodeling defects in yolk sac of Ctf mutants.** A, whole mount images of control (Ctf
fl/fl) and Ctf mutant (Ctf
fl/fl;Tie2-cre) yolk sacs attached to placentae and embryos at E8.5, E9.5, and 10.5. Scale bar = 1 mm. B, immunofluorescence for Pecam-1 (green) on yolk sacs. Lower panels are skeletons generated with AngioTool from fluorescent micrographs. Scale bar = 100 μm. C, quantification of vessel area, total vessel length, and lacunarity from skeletons using AngioTool. Error bars represent the mean ± S.D.* p < 0.05.
might be caused by decreased blood flow; therefore, we analyzed the expression of Nos3 by quantitative PCR (qPCR) and eNOS protein abundance by Western blotting. Nos3 mRNA, and eNOS protein levels were comparable between control and Ctcf mutant yolk sacs at E9.5 (Fig. S3), which agrees with a normally irrigated yolk sac at this stage (Fig. 2A). This suggests that vascular remodeling defects in yolk sac up to E9.5 in Ctcf mutants are not secondary to decreased blood flow because of heart defects or reduced circulating blood.

Ctf is required for placental vascular development

Defective labyrinth development can affect embryonic growth (17, 43). Whole placenta still attached to Ctcf mutant embryos through the umbilical cord appeared normal at E9.5. However, the placenta and umbilical cords in Ctcf mutants appeared improperly irritated at E10.5 (Fig. 3A), suggesting defects in the labyrinth. Accordingly, E9.5 Ctcf mutant placenta had both embryonic and maternal blood vessels. In contrast, only maternal blood vessels were observed in histological sections of E10.5 Ctcf mutant placentae (Fig. 3B). To visualize the labyrinth, we incorporated the cre-dependent GFP reporter Rosa<sup>tm1Gr</sup> (<sup>44</sup>) into Ctcf floxed mice carrying the Tie2-cre transgene (<sup>38</sup>), resulting in GFP-labeled endothelial cells. Quantification on placenta sections stained for GFP revealed a comparable labyrinth area in Ctcf mutants at E9.5, and a significant decrease at E10.5 (Fig. 3, C and D). Decreased labyrinth expansion was not caused by decreased endothelial cell proliferation or increased cell death, as the numbers of endothelial cells positive for phosphorylated histone H3, or activated caspase 3, were comparable between control and Ctcf mutant placentae at E9.5 and E10.5. Caspase 3–positive cells were absent in E10.5 placentae (Fig. S4).

Figure 3. Decreased labyrinth expansion in Ctcf mutants. A, whole control (Ctcf<sup>fl/fl</sup>) and Ctcf mutant (Ctcf<sup>fl/fl;Tie2-cre</sup>) placentae attached to E9.5 and E10.5 embryos. Scale bar = 100 μm. CP, chorionic plate; UM, umbilical cord; EM, embryo. B, histological sections of labyrinth. EB, embryonic blood; MB, maternal blood. Scale bar = 50 μm. C, GFP (green) immunostaining on sections of placentae at E9.5 and E10.5 in control (Ctcf<sup>fl/fl;Tie2-cre;Rosa<sup>tm1Gr</sup></sup>) and mutant (Ctcf<sup>fl/fl;Tie2-cre;Rosa<sup>tm1Gr</sup></sup>) embryos. Scale bar = 200 μm. D, quantification of GFP fluorescence area in E9.5 and E10.5 placentae. Error bars represent the mean ± S.D. NS, nonsignificant; AU, absorbance unit. *, p < 0.05.
protein levels were also comparable between control and Ctf mutant placenta at E10.5 (Fig. S3), suggesting that defective labyrinth expansion is not secondary to heart defects or decreased blood flow. Thus, endothelial Ctcf is required for extraembryonic vascular development.

**Genome-wide expression profile of E9.5 endothelial cells from WT and Ctf mutant placenta**

To uncover genes and pathways regulating vascular development downstream of Ctcf we performed high-throughput RNA-Seq on endothelial cells sorted from placenta of control (Ctcffl/fl;Tie2-cre;RosamT/mG/) and Ctcf mutant (Ctcffl/fl;Tie2-cre;RosamT/mG/) embryos at E9.5. Sorted GFP-positive cells expressed significantly higher levels of GFP and the endothelial markers kinase insert domain receptor (Kdr), and TEK receptor tyrosine kinase (Tek, also known as Tie2), than GFP-negative cells (Fig. S5), indicating that the sorted cell population is enriched for endothelial cells. Consistent with loss of Ctcf protein (Fig. S1, A and B), Ctcf mRNA was drastically reduced in endothelial cells sorted from mutant embryos, as shown by qPCR (Fig. S5B). RNA-Seq analysis revealed 232 genes that were up-regulated, and 155 genes that were down-regulated over 1.5-fold in Ctcf mutant endothelial cells (Fig. 4A). Analysis of misregulated genes using DAVID revealed that up-regulated genes are enriched for processes important for vascular development including focal adhesion, extracellular matrix–receptor interaction, and adherens and tight junction. Downregulated genes are enriched for processes related to cell cycle and genomic stability, and GSH metabolism (Fig. 4B). To validate the RNA-Seq results, we performed qPCR on selected genes that were highly misregulated in Ctcf mutants, including genes with known functions in vascular development. qPCR was performed on endothelial cells sorted from placentae and yolk sacs from control and Ctcf mutant embryos. This analysis confirmed down-regulation of frataxin (Fxn), Gstz1, C3ar1, E2f2, E2f8, and Plt8, and up-regulation of several developmental regulators including Msx1, Tcf7, Celsr1, Ralgds, Pitx1, Tead1, and Notch1 (Fig. 4, C and D and Fig. S6). Changes in gene expression levels in placenta, but not yolk sac, were largely consistent with the RNA-Seq (Fig. 4C), suggesting that placenta and yolk sac endothelial cells have unique gene expression programs, or that Ctcf controls specific transcriptional pathways in endothelial cells in different organs. RNA-Seq revealed that Frataxin (Fxn) was the most down-regulated gene in Ctcf mutant placental endothelial cells. Western blotting revealed a slight but statistically significant decrease in Fxn protein in Ctcf mutant placenta (Fig. 4, E and F). This analysis was carried out on labyrinth tissue; therefore, it likely underestimates protein decrease in endothelial cells. qPCR showed dramatic Fxn down-regulation consistently in both placental and yolk sac endothelial cells (Fig. 4, C and D), suggesting that Fxn might be a Ctcf target important for vascular endothelial cell development.

**CTCF activates the FXN gene promoter**

It has been proposed that CTCF activates FXN expression by maintaining an open chromatin configuration (33, 45–47). Others and we have shown that Ctcf regulates developmental processes by activating gene expression as a transcription factor (1, 3). To determine whether CTCF activates FXN expression as a transcription factor we assessed the capacity of CTCF to activate the FXN promoter in an episomal luciferase reporter. Comparison of the 5′ region immediately upstream the mouse Fxn gene against a database of validated CTCF-binding sites (48, 49) identified a motif spanning nucleotides −4 to −23 relative to the transcription start site (46). This binding site is conserved in the human FNX promoter and has high identity with other previously validated CTCF-binding sites (Fig. 5, A and B). We cloned a 386-bp DNA fragment corresponding to the 5′ regulatory region of the human FNX gene that includes the identified CTCF-binding motif. CTCF significantly activated the FNX promoter in transient cotransfections in bovine aortic endothelial cells (BAECs) (Fig. 5C). Thus, Ctf is a transcriptional activator of FXN in endothelial cells.

**Ctf prevents oxidative stress in endothelial cells**

Frataxin deficiency in yeast (50) and in cells from patients with FRDA causes increased oxidative stress (29, 30), and enhanced oxidative stress is known to negatively affect vascular development (51). Our RNA-Seq analysis revealed that genes down-regulated in Ctf mutant endothelial cells participate in GSH metabolism, including Fxn and GSH S-transferase zeta 1 (Gstz1) (Fig. 4, B and D), which modulate ROS generation (52, 53). This suggests a potential function of Ctcf as an oxidative stress regulator. To determine whether Ctcf deficiency causes oxidative stress in endothelium we analyzed ROS-mediated DNA damage in human umbilical vein endothelial cells (HUVECs) with reduced CTCF levels. CTCF was efficiently knocked down in HUVECs using two nonoverlapping siRNAs (Fig. 5, D and E). CTCF depletion led to decreased levels of FNX mRNA (Fig. 5F). CTCF-depleted cells were stained using an antibody against 8-hydroxyguanosine (8-OHG), a modified base that occurs in DNA as a result of oxidative stress (54). As positive controls, HUVECs with decreased levels of FXN or treated with H2O2, had increased levels of 8-OHG. More CTCF-depleted HUVECs had nuclei that were positive for 8-OHG compared with cells transfected with a control siRNA (Fig. 5, G and H and Fig. S7). Importantly, enhanced oxidative stress was associated with defects in angiogenesis, as CTCF or FXN knockdown (Fig. 5F) cells had decreased tube length in a Matrigel tube formation assay (Fig. 5, I and J).

We assessed whether enhanced oxidative stress was also present in Ctf mutant embryos. In sections of placentae at E10.5, significantly more 8-OHG foci were found in endothelial cell nuclei in Ctf mutant embryos, than in controls (Fig. 6, A and B). Increased ROS promotes lipid peroxidation in a humanized mouse model of FRDA (55). Western blotting revealed increased levels of 4-hydroxynonenal (4HNA), a common byproduct of lipid peroxidation (56), in labyrinth tissue from Ctf mutants compared with controls (Fig. 6, C and D). Furthermore, endothelial cells in Ctf mutant placentae had higher levels of 4HNA than controls (Fig. 6, E and F). Thus, Ctf protects endothelial cells from oxidative stress.

Mitochondrial dysfunction leads to ROS accumulation and lipid peroxidation in a humanized mouse model of FRDA (55). Cytochrome c, an essential component of the mitochondrial electron transport chain indispensable for energy production
is decreased in FXN knockdown and mutant cells (58, 59). Accordingly, Western blot analysis showed that cytochrome c is decreased in labyrinth tissue from Ctf mutant placenta (Fig. 6, G and H). The iron-sulfur cluster assembly enzyme (IscU), which regulates mitochondrial iron homeostasis (60), is also decreased in Fxn mutant mouse tissues (61). Immunofluorescence on sections of labyrinth from control embryos revealed cytoplasmic and nuclear staining for IscU1/2 (IscU1/2) (Fig. 6J). This is consistent with cytoplasmic and nuclear localization of IscU1 in mammalian cells; however, the function of iron-sulfur cluster assembly in nucleus is not clear (62). Immunofluorescence revealed decreased levels of IscU1/2 in endothelial nuclei in placentae of Ctf mutant embryos (Fig. 6, I and J). Thus, similar to FXN-deficient cells, Ctf deficiency leads to a decrease in cytochrome c and IscU proteins.

**Discussion**

The contribution of Ctf to vascular growth during development and postnatally remains poorly understood. Previously, it was shown that Ctf can bind the promoter of Vegf to prevent
surrounding enhancers from activating its expression (15, 63). Accordingly, depletion of Ctcf by shRNA injection in the sub-retinal space causes excess intraretinal vascularization (15). In contrast, we found that Ctcf inactivation in endothelial cells negatively affects embryonic vascular development, and that Vegf expression was not altered in Ctcf mutant endothelial cells in our RNA-Seq and qPCR analysis (Fig. S6). This suggests alternative or context-specific functions for Ctcf in developing vascular endothelium. We found that Ctcf limits oxidative stress in endothelial cells. ROS modulate key signaling pathways controlling vascular development during embryogenesis and regenerative processes (64). Moderate oxidative stress and ROS levels can favor, whereas excessive oxidative stress can be detrimental to, vascular development (51, 65). Our results suggest that Ctcf is an important modulator of oxidative stress that prevents excessive ROS accumulation in endothelial cells to promote vascular development. 

Ctcf is known to affect the proliferation and survival of particular cell types. For example, Ctcf promotes T cell proliferation in the thymus (37). In contrast, Ctcf deficiency in the developing limb or heart does not affect mesenchyme cell (9) or cardiomyocyte (14) proliferation, however, it induces mesenchyme cell apoptosis (9). We found that Ctcf deficiency does not affect proliferation nor does it induce apoptosis in embryonic endothelial cells, suggesting that Ctcf regulates cell growth and maintenance cell specifically. Proliferating endothelial cells produce higher ROS levels than quiescent cells (66). ROS induces activation of signaling pathways that promote endothelial cell proliferation and survival (67). It is possible that increased ROS production might have prevented imbalanced proliferation and apoptosis in Ctcf-depleted endothelial cells at least before E10.5. Alternatively, other mechanisms of cell death, including ferroptosis, might have been affected in Ctcf mutants. Ferroptosis is an iron-regulated route of cell death (68, 69).
dependent on fatty acid synthesis and cysteine transport (70). These processes are linked to 4HNA (70), which was increased in Ctcf mutant placenta (Fig. 6, C–F). To the best of our knowledge, our work demonstrates for the first time that Ctcf regulates ROS accumulation. Future experiments will be required to directly test the extent to which Frataxin is responsible for the oxidative phenotype in Ctcf knockdown endothelial cells. If Ctcf regulates ROS levels in other cell types, it will be of interest to determine whether sensitivity to different ROS levels underlies cell-specific functions of Ctcf.

Decreased ability of cells to relieve oxidative stress has been implicated in cancer, diabetes, and aging and in neurodegenerative (71) and cardiovascular pathogenesis (51, 72). Neurons and cerebellar granule cells from a mouse model of FRDA generate ROS, resulting in decreased GSH (55). In addition, models of frataxin deficiency in yeast, fly, mouse, and cells in culture (73) support a function for frataxin in preventing ROS-induced toxicity in FRDA pathology (74, 75). Accordingly, reducing ROS prevents early mortality in frataxin-deficient Drosophila (76), and improves electrical contraction, coupling, and decay velocity of calcium kinetics in cardiomyocytes derived from stem cells from FRDA patients (77). Endothelial dysfunction has been associated with FRDA (27). However, how oxidative stress in endothelial cells contributes to FRDA has not been investigated. Our finding of decreased frataxin and increased ROS in Ctcf-deficient endothelial cells opens the possibility to investigate the endothelial component of FRDA.
Oxidative stress can potentially alter global gene expression patterns (78). We found that Ctf-depleted developing endothelial cells are under oxidative stress and misregulate hundreds of genes. It will be of interest to determine whether Ctf depletion alters interaction of distal regulatory elements resulting in dysregulated oxidative stress responses in endothelial cells during development. Our results indicate that Ctf is an important regulator of oxidative stress in developing endothelial cells and open the possibility to investigate the endothelial component of diseases associated with oxidative stress.

Experimental procedures

Mice

All animal procedures were approved by the Animal Care Committee at the Hospital for Sick Children and followed the guidelines of The Centre for Phenogenomics. The following strains were used: Ctfβ/β (37), Tie2-cre (38), and ROSA26mT/mG (44). Presence of vaginal plugs indicated E0.5. Embryos not carrying the ROSA26mT/mG transgene were obtained by crossing Ctfβ/+; Tie2-cre males with Ctfβ/β females. Embryos carrying the ROSA26mT/mG transgene were obtained by crossing Ctfβ/+; Tie2-cre males with Ctfβ/β; ROSA26mT/mG females.

Genotyping

Tail clips, ear notches, and yolk sacs were digested in 300 μl of 50 mM NaOH at 95 °C for 10–30 min, and 100 μl of 0.5 μM Tris–HCl were added to neutralize the reaction (79). 1 μl of the digestion was used for PCR. Amplification conditions used to identify floxed Ctf alleles were the following: 94 °C 3 min, 94 °C 30 s, 63 °C 30 s, 72 °C 4 min, 72 °C 2 min, 72 °C 2 min, 72 °C 4 min, 72 °C 35 cycles, and 72 °C for 5 min. Amplification conditions used for Cre were the following: 95 °C 2 min, 95 °C 40 s, 55 °C 50 s, 72 °C 1 min, 72 °C 2 min, 72 °C 4 min, 72 °C 35 cycles, and 72 °C for 10 min. Primers are in Table S1.

Fixation and histology

Freshly dissected embryos in cold PBS were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C. Tissues were washed twice in PBS for 30 min at 4 °C and stored in 70% ethanol overnight at 4 °C. Tissues were washed in the following ethanol and xylene series at room temperature: 85% EtOH for 30 min twice, 95% EtOH for 30 min twice, 100% EtOH for 45 min four times, 100% xylene for 30 min, and 100% xylene for 10 min three times. Tissues were incubated in 50% xylene:paraffin oil at 60 °C for 30 min and stored at room temperature overnight. The next day, tissues were incubated at 60 °C for 30 min, washed with 100% xylene for 1 h at 60 °C twice, and after refreshing the xylene, incubated for 2 h at 60 °C. Tissues were then embedded in wax blocks that were mounted on histology cassettes. 4- to 8-μm-thick sections were generated, mounted on glass slides, and stained with hematoxylin and eosin as follows. Slides were washed in xylene for 10 min twice, 100% EtOH for 2 min twice, 90% EtOH for 2 min, 70% EtOH for 2 min, 50% for 2 min, 30% for 2 min, and quickly washed with tap water three times. Slides were stained with 100% hematoxylin for 10 min, quickly washed three times with tap water, placed in 0.5% acid alcohol for 5 s, quickly washed three times with tap water, placed in 1% lithium carbonate for 5 s, washed with tap water three times, 30% EtOH, 50% EtOH, and 70% EtOH for 1 min. Slides were then stained in 3% eosin for 10 min, washed in 90% EtOH for 1 min, 100% EtOH twice for 1 min, and placed in xylene twice for 5 min. Slides were mounted with Permex (Fisher).

Immunofluorescence and whole mount immunostaining

Dissected tissues were fixed in 4% PFA overnight at 4 °C, washed in PBS three times for 10 min at room temperature, and kept in 30% sucrose/PBS at 4 °C overnight or until tissues sank down. Tissues were embedded in OCT compound and sectioned. 4-μm frozen sections were mounted on glass slides, fixed in 4% PFA for 5 min, and washed in PBS three times, 5 min each. Slides were blocked with 5% goat serum, 0.1% Triton X-100 in PBS for 15 min and incubated with primary antibodies overnight at 4 °C in a humidified chamber. Slides were washed in PBS three times for 10 min each, incubated with secondary antibodies diluted in blocking buffer for 1 h at room temperature. Slides were washed in PBS three times 5 min each and PBS with 0.05% Tween 20 for 5 min. Slides were mounted in Vectashield Mounting Medium with DAPI (Vector Laboratories). Antibodies and dilutions were as follows: Ctf (1/300) (80) (Santa Cruz Biotechnology, G-8), phosphorylated histone H3 (1:100) (Santa Cruz, SC-8656-R), cleaved caspase 3 (1:100) (Cell Signaling Technologies, 9661), CT3 (1:200) (Development Studies Hybridoma Bank), CD31/Pecam-1 (1:100) (BD Pharmingen, 553370), IscU1/2 (1:1000) (Santa Cruz Biotechnology, sc-373694), and GFP (1:1000) (GeneScript, A01694).

Embryos for whole mount immunostaining were fixed in 4% PFA overnight and washed three times in PBS for 5 min each. Embryos were permeabilized and blocked for 1 h at room temperature in PBT (1 × PBS with 0.2% Triton X-100) with 0.1% BSA and 2% goat serum. Embryos were incubated in anti–Pecam-1 antibody diluted in blocking buffer (0.1% BSA and 2% normal goat serum) overnight at 4 °C. Samples were washed five times for 5 min with PBS, blocked in PBT with 2% normal goat serum for 1 h at room temperature, and incubated with secondary antibodies, diluted in 0.15 BSA and 2% goat serum, for 1 h in the dark at room temperature. Samples were washed five times for 5 min each with PBS and cleared in 1:1 glycerol:PBS for 3 h at 4 °C, and then in 80% glycerol:PBS for 1 h at 4 °C before imaging (81).

Cells were transfected at 50% confluence on 10 μg/ml fibronectin-coated Permanox 8-well chamber slides (Thermo Fisher) and allowed to grow to confluence. Cells were then fixed with 4% paraformaldehyde for 20 min, followed by permeabilization for 5 min with 0.2% Triton X-100, and blocking with 5% BSA/PBST for 1 h. Cells were then incubated with mouse anti–8-hydroxyguanosine (1:100) (Santa Cruz Biotechnology), followed by incubation with rabbit anti–mouse IgG 488 (1:200) (Invitrogen), and counterstained and mounted in Vectashield Mounting Medium with DAPI (Vector Laboratories). For actin staining, after incubation with secondary antibody, cells were washed and incubated with rhodamine-phalloidin (1:40) (Invitrogen) for 30 min at room temperature. Images were taken on an Olympus FV1000 Confocal microscope.
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using a LumPlanFl40X/0.8NA objective. The total number of nuclei was divided by the number of nuclei that had an 8-OHG signal.

Gene expression analysis

GFP-positive cells were sorted from control CtcfR+/+;Tie2-cre;Rosa26tm1G and mutant CtcfR/β;Tie2-cre;Rosa26tm1G yolk sacs and placentae, and RNA was isolated using the Direct-zolTM RNA Miniprep kit (Zymo Research). cDNA was synthesized using the SuperScript® VILO cDNA synthesis kit (Thermo Fisher Scientific). cDNA was used in qPCR done using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) on a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad). Data were analyzed using CFX Manager Software (Bio-Rad) and normalized to Pgk1 expression levels. qPCR primer sequences are in Table S1.

RNA-seq

Endothelial cell RNA was isolated from GFP-positive cells (82, 83) sorted from individual dissected placental labyrinth (84) of control CtcfR/++;Tie2-cre;Rosa26tm1G and mutant CtcfR/β;Tie2-cre;Rosa26tm1G embryos using Direct-zolTM RNA Miniprep Plus Kit (Zymo Research) and treated with DNase according to the manufacturer’s protocol. RNA integrity was verified using the Agilent Bioanalyzer (Agilent Technologies). Three biological replicates were used for each group. RNA-Seq libraries were prepared using the Ovation® Single Cell RNA-Seq System (NuGEN Technologies) and sequenced in single-end sequence reads (50 bp in length) on the Illumina HiSeq 2500 platform. The first 8-bp sequences of the 5‘ end of the sequencing reads were trimmed using Trimmomatic (85), as recommended by the Ovation® Single Cell RNA-Seq System (NuGEN) protocol. Trimmed high-quality reads were then mapped to the mouse genome (mm10) using STAR v2.4.2a (86). Mapped read counts were obtained using HTseq (87). Differential expression analysis and MA plots was performed using DESeq2 (88). Enrichment of gene ontology categories in differentially expressed genes was determined using DAVID (89).

Cell culture and transfection

HUVECs and BAECs (ScienCell Research Laboratories) were cultured in Endothelial Cell Medium (ScienCell), 5% fetal bovine serum (FBS), 1% endothelial cell growth supplement (ScienCell), and 1% penicillin/streptomycin. Cells were grown on attachment factor–coated culture dishes and used from passage 3 to passage 6 in experiments. BAECs (Lonza) were cultured in DMEM high glucose (Gibco) with 10% FBS, 1% penicillin/streptomycin. HUVECs were transfected with 40 nM siRNA using RNAi Max (Invitrogen) per manufacturer’s instructions. After 48 h, knockdown was confirmed via qRT-PCR and/or Western blotting.

Tube formation assay

Matrigel (Corning) was polymerized in μ-Slide Angiogenesis chambers (ibidi) for 1 h at 37 °C. siRNA transfected HUVECs were incubated with 5 μM Cell Tracker Green (Thermo Fisher) for 30 min at 37 °C and seeded onto Matrigel for 8 h. Images were taken on a stereo microscope (Leica M165FC), and total tube length was calculated using angiogenesis analyzer (ImageJ). Two fields of view were analyzed per condition and averaged each experiment.

siRNA and plasmids

Nontargeting Silencer Select Negative Control No. 1 siRNA, CTCF siRNA No. 1 and siRNA No. 2 (assay IDs: s20967 and s3855), and Frataxin siRNA (assay ID: s5360) were from Ambion/Invitrogen. The luciferase reporter was constructed by cloning a 386-bp PCR product corresponding to the Frataxin promoter into pGL3 Basic. Primers are in Table S1. The CTCF overexpression plasmid PCI-7.1 was described previously.

Luciferase assays

Confluent BAECs were transfected with 0.5 μg of FXN luciferase construct, 0.5 μg of human CTCF overexpression construct, and 0.1 μg of pRenilla construct using Lipofectamine 2000 (2 μl) in Opti-MEM (Invitrogen). Transfection was performed in 12-well dishes and media were changed back to Endothelial Cell Medium after 5 h. After 24 h, dual luciferase (Renilla and Firefly) was measured using a GloMax 20/20 Luminometer (Promega) using the Dual-Luciferase Reporter Assay System (Promega).

Western blotting

siRNA transfected cells were lysed in 2× Laemelli buffer and boiled at 95 °C for 10 min and centrifuged. Samples were then loaded on precast SDS-PAGE gels (Bio-Rad) for Western blot analysis. Antibodies were used were CTCF (1:500) (Santa Cruz Biotechnology, sc-271474), GAPDH (1:5000) (Santa Cruz Biotechnology, sc-47724), 4 hydroxynonenal (1:200) (Abcam, ab46545), Frataxin (1:100) (Abcam, ab46545), cytochrome C (1:1000) (Santa Cruz Biotechnology, sc-13156), lscU1/2 (1:1000) (Santa Cruz Biotechnology, sc-373694), and HRP-conjugated goat anti-mouse IgG (1:3000) (Cell Signaling Technology, 7076). Blots were processed using MicroChemi 4.2 (DNR Bio-Imaging Systems).

Microscopy and imaging

Nikon SMZ1500 and Nikon Eclipse Ni microscopes were used. Images were analyzed and quantified using ImageJ Cell Counter and Angiogenic Analyzer tools.

Statistical analysis

Data are presented as the mean ± S.D. or S.E., as indicated. Data were compared by Student’s t test. p <0.5 was considered significant. At least three biological replicates were compared in all analyses.

Author contributions—A. R. R. designed and performed experiments, analyzed data, and wrote the manuscript with P. D. O., A. A., L. C., P. V. D., and N. K. designed and performed experiments and analyzed data. J. E. F. designed experiments, analyzed data, and edited the manuscript. M. D. W. conceived the study, designed and performed experiments, analyzed data, and wrote the manuscript with A. R. R with input from all authors.
References

1. Phillips, J. E., and Corces, V. G. (2009) CTCF: Master weaver of the genome. Cell 137, 1194–1211 CrossRef Medline

2. Battistelli, C., Busanello, A., and Maione, R. (2014) Functional interplay between MyoD and CTCF in regulating long-range chromatin interactions during differentiation. J. Cell Sci. 127, 3757–3767 CrossRef Medline

3. Delgado-Olguin, P., Brand-Arzamendi, K., Scott, I. C., Jungholt, B., Stainier, D. Y., Bruneau, B. G., and Recillas-Targa, F. (2011) CTCF promotes muscle differentiation by modulating the activity of myogenic regulatory factors. J. Biol. Chem. 286, 12483–12494 CrossRef Medline

4. Gündör, A., and Ohlsson, R. (2009) Chromosome crosstalk in three dimensions. Nature 461, 212–217 CrossRef Medline

5. Busslinger, G. A., Stocsits, R. R., van der Lelij, P., Axelsson, E., Tedeschi, A., Galjart, N., and Peters, J. M. (2017) Cohesin is positioned in mammalian genomes by transcription, CTCF and Wapl. Nature 544, 503–507 CrossRef Medline

6. Lupiáñez, D. G., Kraft, K., Heinrich, V., Krawitz, P., Brancati, F., Klopacki, E., Horn, D., Kayserili, H., Opitz, J. M., Laxova, R., Santos-Simarro, F., Gilbert-Dussardier, B., Wittler, L., Borschiwer, M., Haas, S. A., et al. (2015) Disruptions of topological chromatin domains cause pathogenic rewiring of gene-enhancer interactions. Cell 161, 1012–1025 CrossRef Medline

7. Narendra, V., Bulajenkov, M., Dekker, J., Mazzoni, E. O., and Reinberg, D. (2016) CTCF-mediated topological boundaries during development foster appropriate gene regulation. Genes Dev. 30, 2657–2662 CrossRef Medline

8. Moore, J. M., Rabaia, N. A., Smith, L. E., Fagerlie, S., Gurley, K., Loukinov, D., Disteche, C. M., Collins, S. J., Kemp, C. J., Lobanenkov, V. V., and Filippova, G. N. (2012) Loss of maternal CTCF is associated with preimplantation lethality of Ctf null embryos. PloS One 7, e34915 CrossRef Medline

9. Soshnikova, N., Montavon, T., Leleu, M., Galjart, N., and Duboule, D. (2010) Functional analysis of CTCF during mammalian limb development. Dev. Cell 19, 819–830 CrossRef Medline

10. Li, T., Lu, Z., and Lu, L. (2004) Regulation of eye development by transcription control of CCCTC binding factor (CTCF). J. Biol. Chem. 279, 27575–27583 CrossRef Medline

11. Herold, M., Barkuhn, M., and Renkawitz, R. (2012) CTCF: Insights into insulator function during development. Development 139, 1045–1057 CrossRef Medline

12. Wan, L. B., Pan, H., Hannenhalli, S., Cheng, Y., Ma, J., Fedoriw, A., Lobanenkov, V., Latham, K. E., Schultz, R. M., and Bartolomei, M. S. (2008) Maternal depletion of CTCF reveals multiple functions during oocyte and preimplantation embryo development. Development 135, 2729–2738 CrossRef Medline

13. Hirayama, T., Tarusawa, E., Yoshimura, Y., Galjart, N., and Yagi, T. (2012) CTCF is required for neural development and stochastic expression of clustered Pcdh genes in neurons. Cell Rep. 2, 345–355 CrossRef Medline

14. Gomez-Velazquez, M., Badia-Careaga, C., Lechuga-Vieco, A. V., Nieto-Arellano, R., Ten, J. J., Rollan, I., Alvarez, A., Torroja, C., Caceres, E. F., Roy, A. R., Galjart, N., Delgado-Olguin, P., Sanchez-Cabo, F., Enriquez, J. A., Gomez-Skarmeta, J. L., and Manzanares, M. (2017) CTCF counter-regulates cardiomyocyte development and maturation programs in the embryonic heart. PLoS Genetics 13, e1006958 CrossRef Medline

15. Tang, M., Chen, B., Lin, T., Li, Z., Pardo, C., Pampo, C., Chen, J., Lien, C. L., Wu, L., Ai, L., Wang, H., Yao, K., Oh, S. P., Seto, E., Smith, L. E., Siemann, D. W., Kladde, M. P., Cepko, C. L., and Lu, J. (2011) Restrained of angiogenesis by zinc finger transcription factor CTCF-dependent chromatin insulation. Proc. Natl. Acad. Sci. U.S.A. 108, 15231–15236 CrossRef Medline

16. Garcia, M. D., and Larina, I. V. (2014) Vascular development and hemodynamic force in the mouse yolk sac. Front. Physiol. 5, 308 CrossRef Medline

17. Watson, E. D., and Cross, J. C. (2005) Development of structures and transport functions in the mouse placenta. Physiology (Bethesda) 20, 180–193 CrossRef Medline

18. Olsson, A. K., Dimberg, Å., Kreuger, J., and Claesson-Welsh, L. (2006) VEGF receptor signaling—in control of vascular function. Nat. Rev. Mol. Cell Biol. 7, 359–371 CrossRef Medline

19. De Val, S., and Black, B. L. (2009) Transcriptional control of endothelial cell development. Dev. Cell 16, 180–195 CrossRef Medline

20. Coultais, L., Chawengaksophak, K., and Rossant, J. (2005) Endothelial cells and VEGF in vascular development. Nature 438, 937–945 CrossRef Medline

21. Fish, J. E., and Wythe, J. D. (2015) The molecular regulation of arteriovenous specification and maintenance. Dev. Dyn. 244, 391–409 CrossRef Medline

22. Demicheva, E., and Crispi, F. (2014) Long-term follow-up of intrauterine growth restriction: Cardiovascular disorders. Fetal Diagn. Ther. 36, 143–153 CrossRef Medline

23. Shaut, C. A., Keene, D. R., Sorenson, L. K., Li, D. Y., and Studler, H. S. (2008) HOXA13 is essential for placental vascular patterning and labyrinthal endothelial specification. PLoS Genetics 4, e100073 CrossRef Medline

24. Santos, R., Lefevere, S., Silvia, D., Seguin, A., Camadro, J. M., and Lesuisse, E. (2010) Friedreich ataxia: Molecular mechanisms, redox considerations, and therapeutic opportunities. Antioxid. Redox Signal. 13, 651–690 CrossRef Medline

25. Nachbauer, W., Boesch, S., Reindl, M., Eigentler, A., Hufler, K., Poewe, W., Löscher, W., and Wanschitz, J. (2012) Skeletal muscle involvement in Friedreich ataxia and potential effects of recombinant human erythropoietin administration on muscle regeneration and neovascularization. J. Neuropathol. Exp. Neurol. 71, 708–715 CrossRef Medline

26. Raman, S. V., Phatak, K., Hoyle, J. C., Pennell, M. L., McCarthy, B., Tran, T., Prior, T. W., Olesik, J. W., Lutton, A., Rankin, C., Kissel, J. T., and Al-Dahkh, R. (2011) Impaired myocardial perfusion reserve and fibrosis in Friedreich ataxia: A mitochondrial cardiomyopathy with metabolic syndrome. Eur. Heart J. 32, 561–567 CrossRef Medline

27. Siassos, G., Gialafos, E., Tousoulis, D., Oikonomou, E., Michalea, S., Kollia, C., Aggeli, C., Maniatis, K., Paraskevopoulos, T., Zisimos, K., Kiofi, S., Papavassiliou, A. G., and Stefanadis, C. (2011) Friedreich ataxia is associated with endothelial dysfunction and increased arterial stiffness. Circulation 124, Suppl. 21, Abstract 9993

28. Patel, P. I., and Isaya, G. (2001) Friedreich ataxia: From GAA triplet-repeat expansion to frataxin deficiency. Am. J. Hum. Genet. 69, 15–24 CrossRef Medline

29. Galk, O., Park, S., Liu, G., Macomber, L., Imlay, J. A., Ferreira, G. C., and Isaya, G. (2006) Mitochondrial iron detoxification is a primary function of frataxin that limits oxidative damage and preserves cell longevity. Hum. Mol. Genet. 15, 467–479 CrossRef Medline

30. Schulz, J. B., Dehmer, T., Schöls, L., Mende, H., Hardt, C., Vorderg, M., Bürk, K., Matson, W., Dichgans, J., Real, M. F., and Bogdanov, M. B. (2000) Oxidative stress in patients with Friedreich ataxia. Neurology 55, 1719–1721 CrossRef Medline

31. Pandey, A., Gordon, D. M., Pain, J., Stemmler, T. L., Dancis, A., and Pain, D. (2013) Frataxin directly stimulates mitochondrial cysteine desulfurase by exposing substrate-binding sites, and a mutant Fe-S cluster scaffold protein with frataxin-bypassing ability acts similarly. J. Biol. Chem. 288, 36773–36786 CrossRef Medline

32. Koutnikova, H., Campuzano, V., Foury, F., Döllé, P., Cazzalini, O., and Koenig, M. (1997) Studies of human, mouse and yeast homologues indicate a mitochondrial function for frataxin. Nat. Genet. 16, 345–351 CrossRef Medline

33. Gottesfeld, J. M., Rusche, J. R., and Pandolfo, M. (2013) Increasing frataxin gene expression with histone deacetylase inhibitors as a therapeutic ap-
Ctcf controls vascular development

proach for Friedreich’s ataxia. J. Neurochem. 126, Suppl. 1, 147–154 CrossRef Medline

34. Rai, M., Soragni, E., Jenessen, K., Burnett, R., Herman, D., Coppola, G., Geschwind, D. H., Gottesfeld, J. M., and Pandolfo, M. (2008) HDAC inhibitors correct frataxin deficiency in a Friedreich ataxia mouse model. Plos One 3, e1958 CrossRef Medline

35. Herman, D., Jenessen, K., Burnett, R., Soragni, E., Perlman, S. L., and Gott-

tesfeld, J. M. (2006) Histone deacetylase inhibitors reverse gene silencing in Friedreich’s ataxia. Nat. Chem. Biol. 2, 551–558 CrossRef Medline

36. Sandi, C., Pinto, R. M., Al-Mahdawi, S., Ezzatizadeh, V., Barnes, G., Jones, S., Rusche, J. R., Gottesfeld, J. M., and Pook, M. A. (2011) Prolonged treat-

ment with pemilic o-aminobenzamide HDAC inhibitors ameliorates the disease phenotype of a Friedreich ataxia mouse model. Neurobiol. Dis. 42, 496–505 CrossRef Medline

37. Heath, H., Ribeiro de Almeida, C., Sleutels, F., Dingjan, G., van de No-
belen, S., Jonkers, I., Ling, K. W., Gribnau, J., Renkawitz, R., Crossfeld, F., Hendriks, R. W., and Galjart, N. (2008) CTCF regulates cell cycle progres-

sion of αβ T cells in the thymus. EMBO J. 27, 2839–2850 CrossRef Medline

38. Proctor, J. M., Zang, K., Wang, D., Wang, R., and Reichardt, L. F. (2005) Vascular development of the brain requires beta integrin expression in the neuroepithelium. J. Neurosci. 25, 9990–9948 CrossRef Medline

39. Sohn, S. I., Sarvis, B. K., Cado, D., and Winoto, A. (2002) ERK5 MAPK regulates embryonic angiogenesis and acts as a hypoxia-sensitive repres-

or of vascular endothelial growth factor expression. J. Biol. Chem. 277, 43344–43351 CrossRef Medline

40. Maltepe, E., Schmidt, J. V., Baunoch, D., Bradfield, C. A., and Simon, M. C. (1997) Abnormal angiogenesis and responses to glucose and oxygen dep-

erivation in mice lacking the protein ARNT. Nature 386, 403–407 CrossRef Medline

41. Zudaire, E., Gambardella, L., Kurcz, C., and Vermeren, S. (2011) A computer-based tool for quantitative analysis of vascular networks. Plos One 6, e27385 CrossRef Medline

42. Lucitti, J. L., Jones, E. A., Huang, C., Chen, J., Fraser, S. E., and Dickinson, M. E. (2007) Vascular remodeling of the mouse yolk sac requires hemode-

myorphic force. Development 134, 3317–3326 CrossRef Medline

43. Cross, J. C. (2005) How to make a placenta: Mechanisms of trophoblast cell differentiation in mice—a review. Placenta 26, Suppl. A, S3–S9 CrossRef Medline

44. Muzumdar, M. D., Tasic, B., Miyamiuchi, K., Li, L., and Luo, L. (2007) A human B lymphoma cell line. Cell Death Differ. 9, 252–263 CrossRef Medline

45. Yan, H., Meng, F., Jia, H., Guo, X., and Xu, B. (2012) The identification and oxidative stress response of a zeta class glutathione S-transferase (GSTZ1) gene from Apis cerana. J. Insect Physiol. 58, 782–791 CrossRef Medline

46. Nakada, Y., Canseco, D. C., Theil, S., Abdusalama, S., Asaithamby, A., San-
tos, C. X., Shah, A. M., Zhang, H., Faber, J. E., Kinter, M. T., Szewda, L. I., Xing, C., Hu, Z., Deberardinis, R. J., Schiattarella, G., Hill, J. A., Oz, O., Lu, Z., Zhang, C. C., Kimura, W., and Sadek, H. A. (2017) Hypoxia induces heart regeneration in adult mice. Nature 541, 222–227 CrossRef Medline

47. Abeti, R., Parkison, M. H., Hargreaves, I. P., Angelova, P. R., Sandi, C., Pook, M. A., Giunti, P., and Abramov, A. Y. (2016) Mitochondrial energy imbalance and lipid peroxidation cause cell death in Friedreich’s ataxia. Cell Death Dis. 7, e2375 CrossRef Medline

48. Esterbauer, H., Schaur, R. J., and Zollner, H. (1991) Chemistry and bio-

chemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic. Biol. Med. 11, 81–128 CrossRef Medline

49. Hütttemann, M., Pecina, P., Rainbolt, M., Sanderson, T. H., Kagan, V. E., Samavati, L., Doan, J. W., and Lee, I. (2011) The multiple functions of cytochrome c and their regulation in life and death decisions of the mam-

malian cell: From respiration to apoptosis. Mitochondrion 11, 369–381 CrossRef Medline

50. Schoenfeld, R. A., Napoli, E., Wong, A., Zhan, S., Reutenauer, L., Morin, D., Buckpitt, A. R., Taroni, F., Lonardel, B., Ristow, M., Puccio, H., and Cortopassi, G. A. (2005) Frataxin deficiency alters heme pathway tran-

scripts and decreases mitochondrial heme metabolites in mammalian cells. Hum. Mol. Genet. 14, 3787–3799 CrossRef Medline

51. Lu, C., and Cortopassi, G. (2007) Frataxin knockdown causes loss of cytoplasmic iron-sulfur cluster functions, redox alterations and induct-

ion of heme transcripts. Arch. Biochem. Biophys. 457, 111–122 CrossRef Medline

52. Tong, W. H., and Rouault, T. A. (2006) Functions of mitochondrial ISCu and cytosolic ISCu in mammalian iron-sulfur cluster biogenesis and iron homeostasis. Cell Metab. 3, 199–210 CrossRef Medline

53. Martelli, A., Wattenhofer-Donze, M., Schmucker, S., Bouvet, S., Reuten-

auer, L., and Puccio, H. (2007) Fratixin is essential for extramitochondrial Fe-S cluster proteins in mammalian tissues. Hum. Mol. Genet. 16, 2651–2658 CrossRef Medline

54. Tong, W. H., and Rouault, T. (2000) Distinct iron-sulfur cluster assembly complexes exist in the cytosol and mitochondria of human cells. EMBO J. 19, 5692–5700 CrossRef Medline

55. Lu, J., and Tang, M. (2012) CTCF-dependent chromatin insulator as a built-in attenuator of angiogenesis. Transcription 3, 73–77 CrossRef Medline

56. Coant, N., Ben Mkaddem, S., Pedruzzi, E., Guichard, C., Tréton, X., Du-
croc, R., Freund, J. N., Cazals-Hatem, D., Bouhnik, Y., Woerther, P. L., Skurnik, D., Grodet, A., Fay, M., Biard, D., Lesuffleur, T., Croc, R., Freund, J. N., Cazals-Hatem, D., Bouhnik, Y., Woerther, P. L., Skurnik, D., Grodet, A., Fay, M., Biard, D., Lesuffleur, T., et al. (2010) NADPH oxidase 1 modulates WNT and NOTCH1 signaling to control the fate of proliferative progenitor cells in the colon. Mol. Cell. Biol. 30, 2636–2650 CrossRef Medline

57. Zhou, Y., Yan, H., Guo, M., Zhu, J., Xiao, Q., and Zhang, L. (2013) Reactive oxygen species in vascular formation and development. Oxid. Med. Cell. Longev. 2013, 374963 CrossRef Medline

58. Peshvariya, H., Dusting, G. J., Jiang, F., Halms, L. R., Sobey, C. G., Drum-

mond, G. R., and Selemidis, S. (2009) NADPH oxidase isoform selective regulation of endothelial cell proliferation and survival. Naunyn Schmie-
debergs Arch. Pharmacol. 380, 193–204 CrossRef Medline

59. Petry, A., Djordjevic, T., Weitnauer, M., Kietzmann, T., Hess, J., and Gör-
lach, A. (2006) NOX2 and NOX4 mediate proliferative response in endo-

thelial cells. Oxid. Med. Cell. Longev. 3, 1473–1484 CrossRef Medline

60. Dixon, S. J., Lemberg, B. M., Lampley, M. R., Skouta, R., Samavati, L., Patcl, D. N., Bauer, A. J., Cantley, A. M., Yang, W. S., Gleason, C. E., Patel, D. N., and Stockwell, B. R. (2012) Ferroptosis: An iron-depen-

dent form of nonapoptotic cell death. Cell 149, 1060–1072 CrossRef Medline

61. Dixon, S. J., and Stockwell, B. R. (2014) The role of iron and reactive oxygen species in cell death. Nat. Chem. Biol. 10, 9–17 CrossRef Medline
70. Dalleau, S., Baradat, M., Guéraud, F., and Huc, L. (2013) Cell death and diseases related to oxidative stress: 4-hydroxynonenal (HNE) in the balance. Cell Death Differ. 20, 1615–1630 CrossRef Medline
71. Lin, M. T., and Beal, M. F. (2006) Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 443, 787–795 CrossRef Medline
72. Panth, N., Paudel, K. R., and Parajuli, K. (2016) Reactive oxygen species: A key hallmark of cardiovascular disease. Adv. Med. 2016, 9152732 CrossRef Medline
73. Perdomini, M., Hick, A., Puccio, H., and Pook, M. A. (2013) Animal and cellular models of Friedreich ataxia. J. Neurochem. 126, Suppl. 1, 65–79 CrossRef Medline
74. Park, S., Gakh, O., Mooney, S. M., and Isaya, G. (2002) The ferroxidase activity of yeast frataxin. J. Biol. Chem. 277, 38589–38595 CrossRef Medline
75. O’Neill, H. A., Gakh, O., Park, S., Cui, J., Mooney, S. M., Sampson, M., Ferreira, G. C., and Isaya, G. (2005) Assembly of human frataxin is a mechanism for detoxifying redox-active iron. Biochemistry 44, 537–545 CrossRef Medline
76. Anderson, P. R., Kirby, K., Orr, W. C., Hilliker, A. J., and Phillips, J. P. (2008) Hydrogen peroxide scavenging rescues frataxin deficiency in a Drosophila model of Friedreich’s ataxia. Proc. Natl. Acad. Sci. U.S.A. 105, 611–616 CrossRef Medline
77. Lee, Y. K., Lau, Y. M., Ng, K. M., Lai, W. H., Ho, S. L., Tse, H. F., Siu, C. W., and Ho, P. W. (2016) Efficient attenuation of Friedreich’s ataxia (FRDA) cardiomyopathy by modulation of iron homeostasis-human induced pluripotent stem cell (hiPSC) as a drug screening platform for FRDA. Int. J. Cardiol. 203, 964–971 CrossRef Medline
78. Lake, R. J., Boetefuer, E. L., Won, K. J., and Fan, H. Y. (2016) The CSB chromatin remodeler and CTCF architectural protein cooperate in response to oxidative stress. Nucleic Acids Res. 44, 2125–2135 CrossRef Medline
79. Vuong, S., and Delgado-Olguin, P. (2018) Mouse genotyping. Methods Mol. Biol. 1752, 1–9 CrossRef Medline
80. Valadez-Graham, V., Razin, S. V., and Recillas-Targa, F. (2004) CTCF-dependent enhancer blockers at the upstream region of the chicken α-globin gene domain. Nucleic Acids Res. 32, 1354–1362 CrossRef Medline
81. Roy, A. R., and Delgado-Olguin, P. (2018) Visualizing the vascular network in the mouse embryo and yolk sac. Methods Mol. Biol. 1752, 11–16 CrossRef Medline
82. Chi, L., Ahmed, A., Roy, A. R., Vuong, S., Cahill, L. S., Caporiccio, L., Sled, J. G., Caniggia, I., Wilson, M. D., and Delgado-Olguin, P. (2017) G9a controls placental vascular maturation by activating the Notch pathway. Development 144, 1976–1987 CrossRef Medline
83. Delgado-Olguin, P., Dang, L. T., He, D., Thomas, S., Chi, L., Sukonnik, T., Khyzha, N., Dobenecker, M. W., Fish, J. E., and Bruneau, B. G. (2014) Ezh2-mediated repression of a transcriptional pathway upstream of Mmp9 maintains integrity of the developing vasculature. Development 141, 4610–4617 CrossRef Medline
84. Chi, L., and Delgado-Olguin, P. (2018) Isolation and culture of mouse placental endothelial cells. Methods Mol. Biol. 1752, 101–109 CrossRef Medline
85. Bolger, A. M., Lohse, M., and Usadel, B. (2014) Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120 CrossRef Medline
86. Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T. R. (2013) STAR: Ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21 CrossRef Medline
87. Anders, S., Pyl, P. T., and Huber, W. (2015) HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics 31, 166–169 CrossRef Medline
88. Love, M. I., Huber, W., and Anders, S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550 CrossRef Medline
89. Huang, D. W., Sherman, B. T., and Lempicki, R. A. (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57 CrossRef Medline