OMA1-mediated integrated stress response protects against ferroptosis in mitochondrial cardiomyopathy

**Highlights**

- Oma1-Dele1-Atf4 signaling elicits the ISR in OXPHOS-deficient hearts
- Loss of Oma1 or Dele1 aggravates mitochondrial cardiomyopathy
- Oma1-Dele1-Atf4 signaling protects against ferroptosis in OXPHOS deficiency
- Oma1-dependent ISR promotes selenium-dependent accumulation of Gpx4

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**In brief**

Ahola et al. demonstrate that OXPHOS deficiency in the heart of Cox10−/− mice causes cardiomyopathy and elicits an integrated stress response along the Oma1-Dele1-Atf4 axis. Oma1-dependent stress signaling preserves the glutathione metabolism and Gpx4 accumulation to limit lipid peroxidation, suppress ferroptosis, and delay cardiomyopathy.
OMA1-mediated integrated stress response protects against ferroptosis in mitochondrial cardiomyopathy

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SUMMARY

Cardiomyopathy and heart failure are common manifestations in mitochondrial disease caused by deficiencies in the oxidative phosphorylation (OXPHOS) system of mitochondria. Here, we demonstrate that the cardiac-specific loss of the assembly factor Cox10 of the cytochrome c oxidase causes mitochondrial cardiomyopathy in mice, which is associated with OXPHOS deficiency, lysosomal defects, and an aberrant mitochondrial morphology. Activation of the mitochondrial peptidase Oma1 in Cox10−/− mice results in mitochondrial fragmentation and induction of the integrated stress response (ISR) along the Oma1-Dele1-Atf4 signaling axis. Ablation of Oma1 or Dele1 in Cox10−/− mice aggravates cardiomyopathy. ISR inhibition impairs the cardiac glutathione metabolism, limits the selenium-dependent accumulation of the glutathione peroxidase Gpx4, and increases lipid peroxidation in the heart, ultimately culminating in ferroptosis. Our results demonstrate a protective role of the Oma1-Dele1-mediated ISR in mitochondrial cardiomyopathy and link ferroptosis to OXPHOS deficiency and mitochondrial disease.

INTRODUCTION

Mitochondrial diseases are rare disorders that together represent the most common group of inherited metabolic diseases with exceptional clinical variety (Fernandez-Vizarra and Zeviani, 2021). Typically, mitochondrial diseases arise from a primary defect in oxidative phosphorylation (OXPHOS), but a general energy deficit is not sufficient to explain the extraordinary cell and tissue specificity in mitochondrial disease. Differences in the various cellular responses to mitochondrial dysfunction appear to, at least partially, determine how cells and tissues cope with mitochondrial stress (Suomalainen and Battersby, 2018). Studies in patients, mouse models, and cultured cells have revealed a conserved transcriptional program that is induced upon mitochondrial dysfunction, termed the integrated stress response (ISR) (Bao et al., 2016; Mick et al., 2020; Quirós et al., 2017; Tyynismaa et al., 2010). A comparative analysis of mouse models with cardiac-specific defects in mitochondrial DNA expression pointed to the activation of the ISR as a common signature in mitochondrial cardiomyopathies (Kühl et al., 2017). Besides metabolic adaptations, impaired OXPHOS activity results in the fragmentation of the mitochondrial network, which allows for the selective removal of damaged mitochondria by mitophagy and preserves cardiac homeostasis (Song et al., 2015). Despite compelling evidence for broad cellular responses to mitochondrial stress, it remains largely enigmatic to what extent these responses represent protective mechanisms or contribute to the progression of mitochondrial disorders.

Mitochondrial proteases govern mitochondrial fitness by degrading misfolded proteins and by balancing mitochondrial dynamics and mitophagy (Ahola et al., 2019; Deshwal et al., 2020). The inner membrane (IM) protease Oma1 is activated upon mitochondrial stress, such as mitochondrial depolarization, oxidative stress, or heat stress, or when misfolded IM proteins accumulate (Baker et al., 2014; Murata et al., 2020; Richter et al., 2015). Oma1 cleaves the dynamin-like GTPase Opa1, which mediates mitochondrial fusion, converting a long form of Opa1 into a short form (Gilkerson et al., 2021; MacVicar and Langer, 2016). Opa1 cleavage upon Oma1 activation limits mitochondrial fusion and causes fragmentation of the mitochondrial network. Genome-wide CRISPR screens identified Dele1 as another substrate that is cleaved by Oma1 upon mitochondrial dysfunction (Fessler et al., 2020; Guo et al., 2020). The stress-induced cleavage of Dele1 by Oma1 leads to the accumulation of Dele1 in the cytosol, where it activates the heme-regulated elf2α kinase (HRI, EIF2AK1) to induce the ISR (Fessler et al., 2020; Guo et al., 2020). HRI is one of several kinases that phosphorylates elf2α (pelf2αx) under different cell stress conditions and turns on the ISR (Bar-Ziv et al., 2020; Mick et al., 2020; Pakos-Zebrucka et al., 2016; Quirós et al., 2017; Tyynismaa...
Figure 1. Oma1 delays the onset of hypertrophic cardiomyopathy in Cox10−/− mice

(A–D) Loss of Cox10 leads to (A) early death of mice, (B) decreased body weight, (C) an increased muscle weight/tibia ratio, and (D) an increased heart/body weight ratio that is shown as percentage. Additional deletion of Oma1 in Cox10−/− mice further aggravates these phenotypes.
et al., 2010), p-eIF2α attenuates global translation and promotes the accumulation and nuclear localization of the transcription factor Atf4, which is rapidly degraded in non-stressed conditions. Atf4 promotes amino acid and one-carbon metabolism, protects against oxidative stress, and induces autophagy (Bao et al., 2016; Pakos-Zebrucka et al., 2016). The ISR is therefore thought to support cellular recovery from stress, but prolonged ISR can activate apoptosis.

The concomitant regulation of Opa1-dependent mitochondrial fusion and Dele1-mediated ISR by Oma1 raises the question of the relative importance of mitochondrial fragmentation and stress signaling in disease. Ablation of Oma1 in mice does not cause gross phenotypes (Quiros et al., 2012) but was found to protect against heart failure (Acap-Perez et al., 2018) and against dilated cardiomyopathy in mice, which lack the mitochondrial protease Yme1l (Wai et al., 2015). Similarly, the loss of Oma1 ameliorated ischemic kidney injury (Xiao et al., 2014) and delayed neurodegeneration in mice lacking prohibitin membrane scaffolds (Korwitz et al., 2016). By contrast, deletion of Oma1 aggravated neurodegeneration in neuron-specific Yme1l–/– mice despite suppressing mitochondrial fragmentation (Sprenger et al., 2019). These results point to cell- and context-specific roles of Oma1 in vivo.

Here, we used cardiac-specific Cox10–/– mice to examine the role of Oma1 in mitochondrial disease caused by OXPHOS deficiency. Cox10 encodes a heme A-farnesyltransferase, which is required for the assembly of the cytochrome c oxidase (COX) complex. Mutations in COX10 are associated with Charcot-Marie-Tooth disease type 1A (Reiter et al., 1997). We demonstrate that Cox10–/– mice develop early-onset lethal cardiomyopathy associated with Oma1-Dele1-Atf4-dependent ISR signaling in the heart, which suppresses ferroptosis and delays mitochondrial cardiomyopathy.

RESULTS

Loss of Oma1 aggravates dilated cardiomyopathy in Cox10–/– mice

To examine the role of Oma1 in mitochondrial OXPHOS disease, we deleted Cox10 and Oma1 genes from skeletal and cardiac muscle by breeding the Cox10fl/fl and Oma1fl/fl animals with mice expressing Cre recombinase under the control of the creatine kinase promoter (Ckmm-Cre). Loss of Cox10 in heart and skeletal muscle led to early-onset dilated cardiomyopathy and death of the mice at a median age of 31 days (Figure 1A). Cox10–/– mice showed growth retardation, progressive loss of skeletal muscle mass, and enlarged hearts, which were accompanied by mild accumulation of connective tissue (Figures 1B–1E). As expected, we observed drastically reduced levels of COX subunits, assembled COX complexes (Figures 1G, S1A, and S1B), and reduced COX activity in the heart lacking Cox10 (Figure 1F). The analysis of cardiac tissue by transmission electron microscopy (TEM) revealed mitochondrial fragmentation and an altered mitochondrial ultrastructure with enlarged and swollen mitochondria and disrupted cristae (Figures 1H and S1C). The loss of Cox10 in the heart leads to the activation of Oma1, as indicated by the increased processing of Opa1 and the accumulation of short Opa1 forms c and e (Figure 1I).

The loss of Oma1 worsened the cardiac phenotype of Cox10–/– mice, whereas the loss of Oma1–/– alone had no apparent effect. Cox10–/– Oma1–/– mice had a shorter lifespan with a median survival of 22 days (Figure 1A) and showed more severe cardiomyopathy and fibrotic lesions indicative of cell death (Figures 1B–1E). The deletion of Oma1 in Cox10–/– mice stabilized long Opa1 forms and decreased mitochondrial fragmentation (Figures 1I and S1C). However, the accumulation of COX subunits and assembled COX complexes was not affected, nor was crista morphogenesis restored in Cox10–/– hearts lacking Oma1 (Figures 1F–1H, S1A, and S1B). These experiments revealed a protective role of Oma1 in severe OXPHOS deficiency in vivo.

LYSOSOMAL DEFECTS IN COX10–/–OMA1–/– CARDIAC MUSCLE

OXPHOS deficiencies impair lysosomal function in various murine tissues and cell models via diverse mechanisms (Baixauli et al., 2015; Civiletto et al., 2018; Demers-Lamarche et al., 2016; Fernandez-Mosquera et al., 2019; Khan et al., 2017). Consistently, we observed large lysosomal structures containing mitochondria and membrane aggregates in Cox10–/– hearts (Figures 2A–2C and S1D). An immunohistochemical analysis showed accumulation of the lysosomal marker Lamp1 and of the autophagic substrate p62/sequesosomes-1 (p62/SQSTM1, hereafter p62) in Cox10–/– hearts (Figures 2D, 2E, S2A, and S2B). Accumulation of p62 partially correlated with Tomm20 staining (Figure S2C). Defective lysosomal structures accumulated further in Cox10–/– Oma1–/– hearts in comparison to Cox10–/– hearts, and also, the steady-state levels of Lamp1 and p62 significantly increased upon loss of Oma1 (Figures 2F and 2G). p62 directs ubiquitinated cargo to autophagic degradation, and accumulation of ubiquitin can be indicative of stalled degradation of damaged proteins (Zientara-Ryter and Subramani, 2019). We detected elevated poly-Ubi levels in Cox10–/– hearts, which were further increased upon ablation of Oma1 (Figure S2D). These results show that mitochondrial dysfunction in Cox10–/–-deficient cardiomyocytes leads to lysosomal defects that worsened in the absence of Oma1.

Mice lacking the COX assembly factor Cox15 in skeletal muscle accumulate aberrant lysosomes and benefit from an enhanced autophagic flux upon mTORC1 inhibition (Civiletto et al., 2018). Since we observed the accumulation of phosphorylated ribosomal protein S6 (pS6) and phosphorylated eukaryotic initiation factor 4E (eIF4E) in heart, we asked whether the loss of Oma1 affects the accumulation of pS6 and eIF4E in Cox10–/– and Oma1–/– hearts (scale bars, 0.5 mm in upper and 50 μm in lower panel).

(F) Enzymatic activity staining of cardiac tissue reveals decreased COX activity in Cox10–/– and Cox10–/– Oma1–/– hearts (scale bars, 100 μm).

(G) Cox10–/– and Cox10–/– Oma1–/– hearts show decreased levels of COX subunits, whereas the levels of other respiratory complex subunits are not affected.

(H) Representative EM images from mouse heart showing distorted mitochondrial ultrastructure in Cox10–/– and Cox10–/– Oma1–/– hearts (scale bars, 2 μm).

(I) Loss of Cox10 activates Oma1 and leads to increased Opa1 processing (different Opa1 isoforms, a–e; I-Opa1, long-Opa1; s-Opa1, short-Opa1).

(A) n = 20 (10 females [f], 10 males [m]), (B) n = 10 (5 f, 5 m), (C) n = 4–6 (f), and (D) n = 10 (5 f, 5 m). Histology n = 5, mixed genders, age 4 weeks. Data represented as mean ± SD. *p < 0.05, **p < 0.01, and ***p < 0.001, unpaired two-tailed Student’s t test and log-rank (Mantel-Cox) test for survival.
Figure 2. Loss of Oma1 in mitochondrial cardiomyopathy leads to lysosomal defects and accumulation of p62.

(A and B) EM images of Cox10<sup>−/−</sup> and Cox10<sup>−/−</sup>Oma1<sup>−/−</sup> hearts.

(C) Quantification of the number of cells containing enlarged lysosomal structures in light microscope images for EM analysis (n = 3 mice).

(legend continued on next page)
binding protein 1 (p4E-BP1), mTORC1 substrates, in Cox10−/− and Cox10−/−Oma1−/− hearts (Figures 2H, 2I, S2E, and S2F), we examined in further experiments whether mTORC1 inhibition upon feeding mice with rapamycin prolongs their lifespan. Rapamycin was detected in hearts of fed wild-type, Cox10−/−, and Cox10−/−Oma1−/− mice by LC-MS analysis (Figure S2G) and inhibited S6 phosphorylation (Figures 2H and 2I). However, feeding with rapamycin did not affect the survival of the mice (Figure 2J) or the heart/body weight ratio (Figure S2H), mitochondrial ultrastructure (Figure 2K), or accumulation of p62 and Lamp1 (Figures 2H, 2I, and S2I–S2K). We therefore concluded that increased mTORC1 signaling does not cause lysosomal deficiencies in these mice and that Oma1 prolongs the lifespan of Cox10−/− mice by another mechanism.

**OMA1 activates the integrated stress response in the Cox10−/− heart**

We performed RNA sequencing (RNA-seq) from mouse heart samples in 4-week-old wild-type, Cox10−/−, Oma1−/−, and Cox10−/−Oma1−/− mice. A pathway analysis identified the eu-karyotic translation initiation factor 2 (eIF2) and 4 (eIF4), mTOR, and nuclear factor erythroid 2–related factor 2 (Nrf2) signaling as the most significantly induced pathways in Cox10−/− hearts (Figure 3A). p53, Atf4, and Nrf2 (also known as Nfe2l2) were the transcription factors that were predicted to be most significantly altered in hearts lacking Cox10, based on their target gene expression (Figure 3B). When comparing the cardiac transcriptome of Cox10−/− and Cox10−/−Oma1−/− mice, we identified Atf4f as the most significantly changed transcription factor, being decreased in the absence of Oma1 (Figure 3C). Together with other transcription factors (Kaspar et al., 2021; Pakes-Zebrucka et al., 2016), Atf4 mediates elf2α signaling in response to different mitochondrial insults as part of the ISR (Condon et al., 2021; Forsström et al., 2019; Mick et al., 2020; Quirós et al., 2017). In agreement with an impaired ISR in Oma1−/− mice, we observed a decreased expression of Atf4 target genes such as Fgf21, Pycr1, and Mthfd2 in Cox10−/−Oma1−/− hearts when compared with Cox10−/− mice (Figure 3D). Notably, rapamycin treatment did not inhibit the ISR in Cox10−/− mice, indicating that signaling does not depend on mTORC1 activation (Figure S3A).

These findings were substantiated by our unbiased TMT-based proteomic analysis of wild-type, Cox10−/−, Oma1−/−, and Cox10−/−Oma1−/− hearts (Figure 3E). Among the 40 most differentially regulated proteins (permutation-based FDR < 0.05) in Cox10−/− hearts, we identified 7 previously described ISR targets (Torrence et al., 2021), which accumulated in Cox10−/− hearts in an Oma1−/− dependent manner (Figure 3E, cluster 4). The steady-state levels of 18 additional proteins were reduced in Cox10−/−Oma1−/− heart proteome but were not altered in Cox10−/−, including proteins involved in the iron-sulfur metabolism (Gpx5), the antioxidant system (Txn2), and several subunits of respiratory complex I harboring iron-sulfur clusters (Figure 3E, cluster 2). Immunoblot analysis confirmed reduced protein levels of Atf4 and of phosphorylated elf2α in Cox10−/−Oma1−/− hearts when compared with Cox10−/− hearts (Figures 3F and 3G), while the levels of Atf5, which mediates the initial stress response to mtDNA maintenance deficiencies in skeletal muscle (Forsström et al., 2019), were not altered in both mouse models (Figure S3B). We conclude that Oma1 is required for the induction of the Atf4-mediated ISR in response to an OXPHOS deficiency in the heart.

**ISR induction in Cox10−/− mice is Dele1 dependent**

To identify possible substrates of Oma1 whose proteolysis contributes to the activation of ISR in Cox10−/− hearts, we compared mitochondrial proteomes of wild-type, Cox10−/−, Oma1−/−, and Cox10−/−Oma1−/− mice (Figure S3C). This analysis revealed that Oma1 facilitates the proteolytic breakdown of likely non-assembled COX subunits in Cox10−/− hearts, but otherwise did not identify candidate Oma1 substrates, whose proteolysis may mediate ISR activation. Although expressed in the heart, we did not detect Dele1 by mass spectrometry, which has been identified as an Oma1 substrate and critical component of Atf4-dependent mitochondrial stress signaling in cultured cells (Fessler et al., 2020; Guo et al., 2020).

We therefore generated Dele1−/− mice by CRISPR-Cas9-mediated genome editing of embryonic stem cells and crossed them with heart and muscle-specific Cox10−/− mice, examining a role of Dele1 for ISR signaling in vivo. Deletion of Dele1 did not broadly affect the survival of mice, which did not show any gross phenotype in the absence of Dele1 up to an age of 1 year (Figure 4A). However, when combined with a deletion of Cox10, Dele1 ablation aggravated the phenotype of Cox10−/− mice, which died at an age of about 4 weeks (Figure 4A). Cox10−/−Dele1−/− mice exhibited a further decreased body weight and increased heart/body weight ratio when compared with Cox10−/− mice (Figure 4B) and further enlarged hearts (Figure 4D). Dele1 ablation did not affect COX activity (Figure 4E). Deletion of Dele1 completely abrogated the accumulation of Atf4 and elf2α in the absence of Cox10 (Figures 4F and 4G) and impaired the expression of ISR target genes (Figure 4H). These results demonstrate that the loss of Dele1 or Oma1 affected phenotypes of heart-specific Cox10−/− mice similarly. The ISR is activated in the heart of Cox10−/− mice along the Oma1–Dele1 axis (Figure 4I), which is associated with a prolonged lifespan of the mice.

**OMA1-mediated ISR response supports the glutathione metabolism in the heart**

The ISR has been shown to inhibit cytosolic translation and promote the expression of genes involved in amino acid synthesis...
Figure 3. Cox10<sup>−/−</sup> mice show Nrf2 and p53 activation and Oma1- and Dele1-mediated Atf4 signaling in cardiac muscle
(A and B) (A) Ingenuity pathway analysis of RNA sequencing results from Cox10<sup>−/−</sup> and wild-type (WT) hearts, showing the 10 most significantly changed pathways and (B) the 5 most significantly changed upstream transcription factors when comparing Cox10<sup>−/−</sup> and WT mice (n = 5).
(C) Heatmap of the ingenuity pathway analysis annotated Atf4 target genes in different mouse lines.
(D) Heatmap of the abundance of 40 proteins, whose steady-state levels were determined by mass spectroscopy and differed most significantly between Cox10<sup>−/−</sup> and Cox10<sup>−/−</sup>Oma1<sup>−/−</sup> hearts.
(F and G) (F) Immunoblot analysis of Atf4 and p-eIF2α in heart lysates from mouse hearts and (G) quantification relative to the loading control (vinculin) and WT. Heatmaps, Euclidian clustering for proteins and samples; Z score of the log2 values; n.a., non-applicable; Ig kappa = chain V-II region 26-10; C1–C4, clusters 1–4.

(Ben-Sahra et al., 2016; Zhang et al., 2018), the one-carbon metabolism (Bao et al., 2016; Dogan et al., 2014; Kühl et al., 2017; Nikkanen et al., 2016; Tyynismaa et al., 2010), and the glutathione (GSH) metabolism (Harding et al., 2003; Torrence et al., 2021). To define the metabolic consequences of an impaired ISR in OXPHOS deficiency, we performed targeted metabolomics in the heart of wild-type, Cox10<sup>−/−</sup>, Oma1<sup>−/−</sup>, and Cox10<sup>−/−</sup>Oma1<sup>−/−</sup> mice (Figures 5A, 5B, S3D, and S3E). We observed the accumulation of one-carbon metabolism intermediates such as serine, glycine, and methionine as well as accumulation of asparagine, alanine, branched-chain amino acids, and proline in Cox10<sup>−/−</sup> hearts (Figure 5A), which is in agreement with ISR induction and previous observations in other mouse models for mitochondrial disease (Kaspar et al., 2021; Kühl...
Figure 4. Loss of Dele1 aggravates mitochondrial cardiomyopathy and impairs the ISR in Cox10−/− heart
(A–C) (A) Loss of Dele1 reduces the lifespan of cardiac-specific Cox10−/− mice, (B) further decreases their body weight, and (C) increases their heart/body weight ratio that is shown as percentage.
et al., 2017; Nikkanen et al., 2016). The deletion of Oma1 did not affect the steady-state level of these metabolites, nor did it strongly impair their accumulation in Cox10−/− hearts (Figure 5A). Similarly, intermediates of the glycolytic and pentose phosphate pathways were present at reduced levels in Cox10−/− hearts regardless of the presence of Oma1 (Figure S3D), while nucleotide levels were not significantly altered in any of the mouse models (Figure S3E). In striking contrast, the accumulation of the reduced form of glutathione (GSH) in Cox10−/− hearts was significantly augmented in Cox10−/− Oma1−/− hearts (Figure 5B), suggesting that Oma1 regulates GSH levels in OXPHOS deficiency.

The GSH metabolism is closely linked to the one-carbon metabolism and is regulated cooperatively by Atf4 and Nrf2 (Figure 5C) (Kasai et al., 2020). Consistent with an impaired ISR in the absence of Oma1, the expression of enzymes involved in GSH metabolism were increased in Cox10−/− hearts, while the expression of several of them was significantly reduced in Cox10−/− Oma1−/− hearts compared with Cox10−/− hearts (Figure 5D). Expression of enzymes that are controlled by Nrf2 and Atf4 together was increased in Cox10−/− Oma1−/− hearts. Moreover, our proteomic analysis revealed the accumulation of many of these enzymes in Cox10-deficient hearts and lower steady-state levels in the absence of Oma1 (Figure 5E). These proteins include the GSH-dependent lipid peroxidase Gpx4, the GSH-degrading enzyme Chac1 and Chop1, whose accumulation was also monitored by immunoblot analysis of heart tissues (Figures 5F and 5G). Chop1 modulates Atf4 activity (Kaspar et al., 2021) and, together with Atf4, has been shown to regulate Chac1 expression to induce apoptosis in prolonged ISR (Mun et al., 2021) and, together with Atf4, has been shown to regulate GSH metabolism in Cox10−/− hearts (Figure S3F).

**A ferroptotic signature of Cox10−/− Oma1−/− hearts**

While acute ISR promotes metabolic reprogramming supporting cell survival, persistent ISR can trigger apoptotic cell death (Bar-Ziv et al., 2020; Galenár et al., 2010; Lange et al., 2008). Unsupervised clustering of our heart RNA-seq data for genes associated with cell death revealed differential regulation of these genes in Cox10−/− and Cox10−/− Oma1−/− hearts, including the pro-apoptotic proteins Chop and Chac1 (Figures 6A and S4A). TUNEL staining of heart sections, which detects DNA fragmentation in apoptotic cells, showed more TUNEL-positive cardiomyocytes in Cox10−/− than in Cox10−/− Oma1−/− hearts (Figures 6B and 6C). However, the low number of the detected TUNEL-positive cells unlikely explains the severity of cardiomyopathy in Cox10−/− hearts, nor the aggravated phenotype in Cox10−/− Oma1−/− mice. Moreover, our gene expression analysis pointed to an inflammatory response specifically in Cox10−/− Oma1−/− hearts (Figures 6D and S4A), while apoptosis is generally considered as a non-inflammatory form of cell death. We therefore reasoned that a different form of cell death may explain the increased vulnerability of cardiomyocytes in Cox10−/− Oma1−/− mice.

The accumulation of GSH and the reduced steady-state level of Gpx4 point to an impaired redox homeostasis in Cox10−/− Oma1−/− mice. Gpx4 limits lipid peroxidation and protects cells against ferroptosis, a non-apoptotic form of cell death driven by iron-dependent phospholipid peroxidation (Dixon and Stockwell, 1999; Gan, 2021; Jiang et al., 2021). We therefore monitored the accumulation of malondialdehyde (MDA), the degradation product of lipid peroxidation, in Cox10−/− and Cox10−/− Oma1−/− hearts (Figures 6E and 6H). MDA-positive areas were detected in Cox10−/− hearts but significantly accumulated in Cox10−/− Oma1−/− hearts (Figures 6E and 6G). Oma1−/− hearts showed wild-type levels of MDA (Figure S4B). Immunostaining with antibodies directed against 4-hydroxy-2-nonenal (4-HNE), a product of endogenous lipid peroxidation, confirmed these findings (Figure S4C). Increased lipid peroxidation correlated with the appearance of lipid swirls detected in TEM images (Figure 5F), raising the possibility that these structures represent oxidized mitochondrial membranes. We conclude from these experiments that the loss of Oma1 is associated with an inflammatory response, the accumulation of GSH, reduced Gpx4 levels, and increased lipid peroxidation in the hearts of Cox10−/− mice, which thus show hallmarks of ferroptosis.

To provide further evidence that these phenotypes result from an impaired ISR in the absence of Oma1, we also examined the lipid peroxidation in the hearts of Cox10−/− Dele1−/− mice by MDA staining (Figures 6G and 6I). Deletion of Dele1−/− caused increased lipid peroxidation in Cox10−/− mice (Figures 6G and 6I), as observed upon ablation of Oma1 (Figure 6E). Moreover, similarly to Cox10−/− Oma1−/− mice (Figures 5E–5G), loss of Dele1 in Cox10−/− hearts decreased Gpx4 protein levels to wild-type levels (Figure 6J). Thus, the deletion of either Oma1 or Dele1, abrogating the ISR in Cox10−/− hearts, is associated with decreased Gpx4 protein levels and increased lipid peroxidation, indicating that the Oma1-Dele1-Atf4 signaling axis protects against ferroptosis in OXPHOS-deficient hearts (Figure 6K).

**The Oma1-dependent ISR protects against ferroptosis in vitro**

To unambiguously demonstrate the protective role of ISR signaling against ferroptosis, we isolated MEFs from wild-type, Cox10−/−, Oma1−/−, and Cox10−/− Oma1−/− mice. We observed a significantly decreased survival of Cox10−/− Oma1−/− cells in vitro when compared with wild-type, Cox10−/−, or Oma1−/− cells (Figures 7A, 7B, and S4D), which was suppressed in the presence of the antioxidant ferrostatin-1 (Figure 7C). This suggests ferroptosis of Cox10−/− Oma1−/− cells, since ferrostatin-1...
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inhibits iron-dependent lipid peroxidation (Miotto et al., 2020). Consistently, the Rbp1 kinase inhibitor necrostatin-1 or the caspase inhibitor QVD, which impairs necroptosis and apoptosis, respectively, had only a minor effect on the survival of Cox10<sup>−/−</sup> Oma1<sup>−/−</sup> cells (Figure 7C). Ferroptosis of Cox10<sup>−/−</sup> Oma1<sup>−/−</sup> cells was accompanied by the increased expression of inflammatory marker genes, which was at least to some extent suppressed in the presence of ferrostatin-1 (Figure S4E). We also observed an increased vulnerability of Cox10<sup>−/−</sup> Oma1<sup>−/−</sup> cells for ferroptosis, which was induced by erastin, an inhibitor of the cystine-glutamate antiporter Slc7a11 (system xc<sup>−</sup>) (Figures 7D and 7E). These experiments demonstrate that Oma1 protects Cox10<sup>−/−</sup> MEFs against ferroptosis.

Similarly to cardiomyocytes in vivo, the loss of Cox10 in MEFs induced the ISR in an Oma1-dependent manner. We observed the accumulation of Atf4 in Cox10<sup>−/−</sup> but not Cox10<sup>+/+</sup> Oma1<sup>−/−</sup> MEFs when cells were grown in oxidative galactose media (Figure 7F). This was accompanied by an increased and Oma1-dependent expression of Atf4 target genes, including various enzymes involved in the metabolism of GSH (Figure 7G). Notably, Gpx4 protein levels were increased in Cox10<sup>−/−</sup> cells but not in Cox10<sup>−/−</sup> Oma1<sup>−/−</sup> cells (Figure 7F). The accumulation of Atf4, Chop, and the expression of the ISR target gene Fgf21 in Cox10<sup>−/−</sup> cells also depended on Dele1 (Figures 7H, S4F, and S4G). We therefore conclude that the loss of Cox10 in MEFs activates the ISR along the Oma1-Dele1 axis, recapitulating our findings in cardiomyocytes in vivo.

Metabolic analysis of wild-type, Cox10<sup>−/−</sup>, Oma1<sup>−/−</sup>, and Cox10<sup>−/−</sup> Oma1<sup>−/−</sup> MEFs revealed the accumulation of cysteine, cystathionine, and GSH in Cox10<sup>−/−</sup> but not Cox10<sup>−/−</sup> Oma1<sup>−/−</sup> cells (Figures 7I and S4H). The increased pool of these metabolites correlates with increased ISR signaling in Cox10<sup>−/−</sup> cells and likely serves as a reservoir protecting Cox10<sup>−/−</sup> cells against oxidative damage and erastin-induced ferroptosis (Figures 7D and 7E). Oma1 did not affect ROS production, nor iron accumulation, which was indistinguishable between Cox10<sup>−/−</sup> and Cox10<sup>−/−</sup> Oma1<sup>−/−</sup> MEFs (Figures S4I and S4J).

To demonstrate the protective role of ISR against ferroptosis, we treated Cox10<sup>−/−</sup> cells with ISRIB, an inhibitor of eIf2α phosphorylation and ISR signaling (Sidrauski et al., 2013) before we examined the vulnerability of the cells for erastin-induced ferroptosis. Short treatment with ISRIB increased the susceptibility of wild-type cells to erastin, whereas the survival of Cox10<sup>−/−</sup> was not affected under these conditions (Figure 7J), likely due to the increased levels of cysteine and GSH in these cells. However, we observed increased erastin-induced ferroptosis in Cox10<sup>−/−</sup> cells upon prolonged ISR inhibition with ISRIB (Figure 7J). Similarly, depletion of Atf4 or Dele1 increased the vulnerability of Cox10<sup>−/−</sup> cells for erastin-induced ferroptosis (Figures 7K and S4K). These experiments demonstrate a protective effect of the Oma1-Dele1-mediated ISR against ferroptosis.

Oma1 supports selenium utilization to promote Gpx4 accumulation

ISR signaling regulates the one-carbon metabolism as well as the metabolism of amino acids and of GSH, broadly promoting the oxidative defense. Gpx4 has been identified as central lipid peroxidase protecting against ferroptosis. However, although impaired ISR signaling caused decreased Gpx4 protein levels in Cox10<sup>−/−</sup> hearts (Figures 5F and 5G), transcription of Gpx4 was not altered in Cox10<sup>−/−</sup> Oma1<sup>−/−</sup> hearts when compared with Cox10<sup>−/−</sup> hearts (Figure 5D), indicating that Gpx4 is not a transcriptional target of Atf4 in cardiomyocytes. Gpx4 is a selenoprotein (Ingold et al., 2018), whose translation depends on the availability of selenium (Li et al., 2022). The ISR is known to affect the trans-sulfuration pathway (Figure 5C) (Suomalainen and Battertsby, 2018), and the involved enzymes have been demonstrated to allow trans-snylation (Lazard et al., 2015). These enzymes include the cystathionine γ lyase Cth, which provides selenophosphate for the synthesis of selenocysteine (Figure 5C) and whose increased expression in Cox10<sup>−/−</sup> hearts depended on Oma1 (Figure 5D). To examine whether the selenium availability limits the accumulation of Gpx4, we treated wild-type, Cox10<sup>−/−</sup>, Cox10<sup>−/−</sup> Oma1<sup>−/−</sup>, and Oma1<sup>−/−</sup> cells with selenium and monitored Gpx4 protein levels (Figure 7L). Strikingly, we observed significantly increased Gpx4 levels in wild-type cells supplemented with sodium selenite, whereas Gpx4 was only moderately increased in Oma1<sup>−/−</sup> cells (Figure 7L). Loss of Oma1 in Cox10<sup>−/−</sup> cells also reduced the selenium-dependent accumulation of Gpx4, although to a lower extent (Figure 7L). Depletion of the Atf4 target Cth prevented Gpx4 accumulation in Cox10<sup>−/−</sup> cells (Figure 7M). In agreement with recent findings in breast cancer cells (Li et al., 2022) and the observed Gpx4 protein levels, selenium supplementation reduced ferroptosis of wild-type MEFs, while the protective effect was ameliorated in Oma1<sup>−/−</sup> cells (Figure S4L).

Thus, the Oma1-mediated ISR increases the transcription of enzymes of the trans-sulfuration pathway, which supports the utilization of selenium and thereby promotes the accumulation of Gpx4 and resistance against lipid peroxidation and ferroptosis (Figure 7N).

**DISCUSSION**

Our results demonstrate that Oma1-Dele1-dependent ISR signaling serves a protective function against ferroptosis and...
Figure 6. Cox10^{-/–} mice show an Oma1-dependent ferroptotic signature and inflammation in the heart
(A) Heatmap of mRNA levels of genes related to innate immune processes (GO term) in the heart.
(B and C) (B) TUNEL staining of mouse heart sections and (C) quantification-positive cells (n = 5 mice) (scale bars, 50 μm).

(legend continued on next page)
delays cardiomyopathy. The ISR promotes the one-carbon metabolism, which supports GSH synthesis via the trans-sulfuration pathway and increases the cellular resistance against oxidative stress (Harding et al., 2003; Torrence et al., 2021). We demonstrate that trans-sulfuration and the GSH metabolism are important metabolic targets of the ISR protecting OXPHOS-deficient cardiomyocytes against ferroptosis. Impaired ISR signaling in Cox10−/−/Oma1−/− hearts disturbs the GSH metabolism and results in decreased Gpx4 levels, which together explain the increased lipid peroxidation and ferroptosis in these mice. Our findings therefore reveal the physiological relevance of the observed strong synthetic interaction of Gpx4 with mitochondrial dysfunction in genome-wide CRISPR screens (To et al., 2019). The Oma1-dependent increased transcription of enzymes of the trans-sulfuration pathway, such as Cth, in the heart supports the utilization of selenium and the synthesis of the selenoprotein Gpx4. Our experiments in cultured cells indicate that an impaired ISR limits available selenium pools and Gpx4 translation in cardiomyocytes, increasing their ferroptotic vulnerability.

Oxidative stress in mitochondria activates the Nrf2-mediated stress response that together with Atf4 induces GSH synthesis and antioxidant defense (Kasai et al., 2020). We observed increased expression of Nrf2 target genes in both Cox10−/− and Cox10−/−/Oma1−/− mice but only partial induction of the GSH defense system without Oma1-mediated Atf4 activation in Cox10−/−/Oma1−/− hearts. While we detected Atf4 and Nrf2 activation in OXPHOS-deficient heart, mild OXPHOS deficiencies in mtDNA replication-deficient skeletal muscle signal via Atf5 and manifest with a disbalance of one-carbon metabolites and nucleotides (Forström et al., 2019; Nikkanen et al., 2016). Mitochondrial translation defects in heart and skeletal muscle lead to Atf4- and Atf5-mediated stress responses (Dogan et al., 2014). It thus appears that the combined and timely controlled expression of different transcription factors in defined metabolic settings determines the stress response in a tissue-specific manner.

Although persistent ISR signaling can induce apoptosis and although Oma1 protects against apoptosis under defined conditions in vitro (Anand et al., 2014; Jiang et al., 2014), we only observed apoptotic cell death of a low number of cardiomyocytes in vivo, unlikely driving cardiomyopathy. Indeed, the mitochondrial apoptotic machinery is age regulated both in mice and human and downregulated in the first weeks of life in mice (Sarosiek et al., 2017). OXPHOS deficiency can increase ROS production and decrease the levels of coenzyme Q (Kühl et al., 2017), which serves protective functions against lipid peroxidation and ferroptosis (Bersuker et al., 2019; Doll et al., 2019). It is conceivable that this increases the dependency of OXPHOS-deficient cardiomyocytes on ISR signaling and renders them susceptible for ferroptosis. Our results provide rich in vivo support for ferroptosis in mitochondrial cardiomyopathies, adding to the emerging evidence for important roles of ferroptosis in cardiovascular diseases in general (Li et al., 2021; Tadokoro et al., 2020).

We observed increased Opa1 processing by Oma1 and mitochondrial fragmentation in Cox10−/− mice. However, although ablation of Oma1 stabilizes long Opa1 forms, it does not ameliorate mitochondrial defects or cardiomyopathy in these animals. However, our results do not exclude that the fragmentation of the mitochondrial network contribute to cardiomyopathy. Indeed, disturbances in mitochondrial morphology have been linked to heart disease (Dorn, 2015). Thus, the role of both Oma1-dependent pathways—ISR signaling and the regulation of mitochondrial dynamics—for cell survival and their relative pathophysiologial importance appear to vary between different tissues and to depend on the metabolic state of the cell. This variability may contribute to the cell- and tissue-specificity in mitochondrial disease.

**Limitations of study**

Deletions of either of the two key components of mitochondrial ISR signaling phenocopy each other and aggravate cardiomyopathy in Cox10−/− mice with hallmarks of ferroptosis. These findings correlate with our experiments in cultured cells, which reveal the protective function of the ISR against ferroptosis. To unambiguously demonstrate that the activation of ferroptosis aggravates cardiomyopathy in vivo requires us to pharmacologically suppress ferroptosis using peritoneal injections. However, these experiments are hampered by the early-onset phenotype of cardiac-specific Cox10−/− mice, offering only a short therapeutic window for treatments of newborn mice. Another limitation of our study concerns other possible substrates of Oma1, whose impaired proteolysis may contribute to mitochondrial cardiomyopathy. Substrate-specific mouse models will be required to delineate the role of individual substrate proteins for cardiac health.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability

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(D) Heatmap of mRNA levels of genes related to cell death processes (GO term) in the heart.

(E and F) Malondialdehyde (MDA) staining of heart sections of WT, Cox10−/−, and Cox10−/−/Oma1−/− mice (scale bars, 20 μm) and (F) EM images showing accumulation of membranous swirls (scale bars, 2 μm).

(G) Malondialdehyde (MDA) staining of heart sections of WT, Cox10−/−, and Cox10−/−/Dele1−/− mice (scale bars, 20 μm).

(H and I) Quantification of the total MDA staining areas (n = 5 animals).

(J and K) (J) Immunoblot analysis of Gpx4 protein levels and (K) quantification relative to vinculin.

(L) Schematic picture of the Oma1-Dele1-mediated ATF4 activation supporting GSH synthesis and antioxidant defense to protect cells from ferroptosis. Heatmaps, Euclidian clustering for genes and samples, Z score of the log, values; C1–C3, clusters 1–3. Data represented as mean ± SD.

*p < 0.05, **p < 0.01, and ***p < 0.001, unpaired two-tailed Student’s t test. Brightness and contrast altered in Figures 5B and 5E images for visualization equally.
Figure 7. ISR activation along the Oma1-Dele1-Atf4 axis protects Cox10−/− MEFs against ferroptosis
(A) YOYO-1-positive, dead WT, Cox10−/− (CKO) and Cox10−/− Oma1−/− (COKO) and Oma1−/− (OKO) MEFs accumulating in relation to cell confluency (n = 4).
(B) Representative images of dead cells stained with YOYO-1 in an IncuCyte analyser (scale bars, 100 μm).
(legend continued on next page)
**METHOD DETAILS**

- Mouse breeding and tissue collection
- Electron microscopy
- Protein Digestion for proteomics
- Liquid Chromatography and Mass Spectrometry for heart proteomics
- Analysis of proteomic data
- Metabolite extraction of polar and lipophilic metabolites from mouse heart tissues
- Metabolite extraction of polar and lipophilic metabolites from MEFS
- Targeted liquid chromatography-high-resolution mass spectrometry-based (LC-HRS-MS) analysis of amine-containing metabolites from mouse heart tissue and MEF cells cultures
- Liquid Chromatography-High Resolution Mass Spectrometry-based (LC-HRMS) analysis of rapamycin
- Anion-Exchange Chromatography Mass Spectrometry (AEX-MS) for the analysis of anionic metabolites from mouse tissue
- Data analysis of LC-MS metabolites
- Cell culture, transfection and RNA interference
- FACS analysis
- Live-cell immunofluorescent assay
- RNA sequencing and quantitative PCR with reverse transcription
- SDS–PAGE, Blue-native and immunoblot analysis
- Immunofluorescence and histological staining

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.cmet.2022.08.017.

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**AUTHOR CONTRIBUTIONS**

Conceptualization, S.A., P.R.M., and T.L.; methodology and formal analysis, H.N. and P.G.; investigation, S.A., P.R.M., S.H., and S.C.; writing, S.A. and T.L.; funding acquisition, S.A. and T.L.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**(C)** Accumulation of dead WT and COKO cells in the presence of ferrostatin (Fer-1), necrostatin (Nec-1) or pan-caspase inhibitor QVD (n = 3–4).

**(D and E)** Representative images of dead cells stained with YOYO-1 in an IncuCyte analyser (scale bars, 100 μm).

**(F)** Accumulation of Atf4, Chop, and Gpx4 in MEFS grown for 22 h in the presence of galactose and quantification of protein levels relative to the loading (vinculin) and WT (n = 6).

**(G)** mRNA levels of Atf4 target genes in galactose-treated MEFS (n = 3).

**(H and I)** Accumulation of Atf4 and Chop in galactose-treated WT and CKO MEFS upon depletion of Dele1 for 72 h and (I) cystathionine and cysteine levels in the indicated MEFS in relation to protein amount and WT (n = 5).

**(J)** Dead, YOYO-1-positive cells accumulating in relation to conflueney upon erastin treatment (16 h) after a pre-treatment for 24 h (short-term) or for 7 days (long-term) with ISRIB (n = 4).

**(K)** Dead, YOYO-1-positive cells accumulating in relation to conflueney upon erastin treatment (16 h) after siRNA-mediated depletion of Dele1 for 72 h (n = 3).

**(L and M)** Accumulation of Gpx4 in MEFS that were pre-treated with sodium selenite (24 h) and then grown in galactose (16 h). Quantification of protein levels relative to loading (tubulin) and WT. (M) Accumulation of Gpx4 in MEFS upon knockdown of Cth or incubation with scrambled control (48 h). After incubating cells on galactose (16 h), protein levels were quantified relative to the loading control (tubulin) and WT.

**(N)** Schematic illustration of the Oma1-Dele1-Atf4 signaling activating trans-sulfuration and GSH metabolism to promote Gpx4 synthesis.

Data represented as mean ± SD or (L) as violin blot with min and max values.

*p < 0.05, **p < 0.01, and ***p < 0.001, unpaired two-tailed Student’s t test.

14 Cell Metabolism 34, 1–17, November 1, 2022
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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| OPA1                | BD     | Cat# 612607; RRID: AB_399889 |
| SDHA                | Abcam  | Cat# ab14715; RRID: AB_301433 |
| VINCULIN            | CST    | Cat# 4650; RRID: AB_10559207 |
| OXPHOS              | Abcam  | Cat# ab110413; RRID: AB_2629281 |
| SQSTM1/p62          | Abnova | Cat# H0000878-M01; RRID: AB_437085 |
| LAMP1               | Novus  | Cat# NB120-19294; RRID: AB_788858 |
| Phospho-4E-BP       | CST    | Cat# 2855; RRID: AB_560835 |
| Phospho-S6          | CST    | Cat# 2211L; RRID: AB_331679 |
| ATF4                | CST    | Cat# 11815; RRID: AB_261025 |
| ATF5                | Abcam  | Cat# ab184923; RRID: AB_280462 |
| Phospho-eIF2alpha   | Abcam  | Cat# ab32157; RRID: AB_732117 |
| CHOP/DDIT3          | CST    | Cat# 2895; RRID: AB_2089254 |
| CHAC1               | Bioss Antibodies | Cat# Bs-6795R |
| GPX4                | Abcam  | Cat# ab105066; RRID: AB_10714549 |
| COX4                | Thermo | Cat# A21348; RRID: AB_2535839 |
| NDUF4               | Invitrogen | Cat# 459100; RRID: AB_2532223 |
| Malondialdehyde, MDA | Abcam | Cat# ab243066 |
| 4HNE                | Millipore | Cat# AB5605; RRID: AB_569332 |
| Poly-Ubi            | CST    | Cat# 3936; RRID: AB_331292 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| ISRIB               | Sigma  | SML0843    |
| YOYO-1 Iodide       | Invitrogen | Y3601    |
| TUNEL assay kit -BrdU | Abcam | ab66110 |
| Masson’s trichrome  | Abcam  | ab150686   |
| Erastin             | Sigma  | E7781      |
| Ferrostatin 1       | Sigma  | SML0583    |
| Sodium Selenite     | Sigma  | S5261      |
| QVD                 | Sigma  | SML0063    |
| GoScript Reverse Transcription Mix | Promega | A2791 |
| PowerSYBR Green PCR Master Mix | Thermo | 4368708 |
| MitoSOX Red Mitochondrial Superoxide Indicator | Thermo | M36008 |
| MitoTrackerDeep Red | Invitrogen | M22426 |

### Deposited data

| NATURE OF DEPOSITED DATA | SOURCE |
|--------------------------|--------|
| Whole cell proteome from mouse heart | This paper | ProteomeXchange: PXD031641 |
| Metabolome data from mouse heart | This paper | https://doi.org/10.5281/zenodo.6943298 |

### Experimental models: Cell lines

| Cell line                        | SOURCE | IDENTIFIER |
|----------------------------------|--------|------------|
| Immortalized Mus musculus embryonic fibroblasts, Cox10fr/wt and Cre treated (KO, Cox10fr/wt) | This paper | N/A |
| Immortalized Mus musculus embryonic fibroblasts, Cox10fr/wt Oma1fr/wt and Cre treated (KO, Cox10fr/wt Oma1fr/wt) | This paper | N/A |
| Immortalized Mus musculus embryonic fibroblasts, Cox10fr/wt and Cre treated (WT) | This paper | N/A |
| Immortalized Mus musculus embryonic fibroblasts, Oma1fr/wt and Cre treated (KO, Oma1fr/wt) | Anad et al. (2014) | N/A |

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### RESOURCE AVAILABILITY

#### Lead contact
Further information and requests for reagents may be directed to and will be fulfilled by the lead contact, Prof. Thomas Langer, PhD (tlanger@age.mpg.de).

#### Materials availability
Mouse and cell lines generated in this study will be shared by the lead contact upon request with MTA.

#### Data and code availability
- Heart RNA-seq data have been deposited at SRA, heart proteome data at ProteomeXchange (PRIDE) and metabolome data at Zenodo. Accession numbers are listed in the key resources table. Original western blot images reported in this paper are included in Data S1 and numerical values for all graphs in Data S1.
- This paper does not report original code
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

#### METHOD DETAILS

### Mouse breeding and tissue collection
All animal work was approved by local authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Germany) and animal procedures were carried out in accordance with European, national and institutional guidelines and according to good practice of animal handling. Mice were maintained at the specific-pathogen-free animal facility of the Max Planck Institute for Biology of Ageing and CECAD Research Centre with 12 h light cycle and regular chow diet. Mice were fed with rapamycin chow (42 mg/kg, ssniff Spezialdiäten) from weaning until the sacrifice of the animals. Rapamycin was purchased from LC Laboratories (Cat. No. R-5000) and encased with Eudragit S100 (Evonik CYRO LLC, Product No. 1-207-490-4242)) by Southwest Research.
Electron microscopy

1-2 mm piece from heart tissue was fixed in 2% formaldehyde/2% glutaraldehyde in 0.1 M cacodylic acid at least 48 h at 4°C. Samples were then washed twice 15 min in 0.1 M cacodylic acid and fixed with 2% osmiumtetroxid (Science Services) in 0.1 M cacodylic acid and washed again twice 15 min in 0.1 M cacodylic acid. After changes in ethanol 50%-100%, a mixture ethanol/propyleneoxid and 100% propyleneoxid the tissue was embedded in Epon fixative. Fixed tissue was cut in 70 nm sections. Samples were then washed four times 15 min in 0.1 M cacodylic acid and fixed with 2% osmiumtetroxid (Science Services) in 0.1 M cacodylic acid and washed again twice 15 min in 0.1 M cacodylic acid. After changes in ethanol 50%-100%, a mixture ethanol/propyleneoxid and 100% propyleneoxid the tissue was embedded in Epon fixative. Fixed tissue was cut in 70 nm sections.

Protein Digestion for proteomics

For lysis of heart samples, 4% SDS in 100 mM HEPES pH = 8.5 was used as a buffer system and the Precellys tissue homogenizer was utilized for mechanical disruption of the tissue following the manufacturer’s instructions. The protein concentration was determined. 10 µg of protein was subjected for tryptic digestion. Proteins were reduced (10 mM TCEP) and alkylated (20 mM CAA) in the dark for 45 min at 45°C. Samples were subjected to SP3 based digestion including a pooled set consisting of all heart samples utilized which served as an internal standard for each TMT batch (n=2 using 126 Channel). Washed SP3 beads (SP3 beads (Sera-Mag (TM) Magnetic Carboxylate Modified Particles (Hydrophobic), Sera-Mag (TM) Magnetic Carboxylate Modified Particles (Hydropolylic) from Thermo Fisher Scientific) were mixed equally, and 3 µL of bead slurry were added to each sample. Acetonitrile was added to a final concentration of 50% and washed twice using 70 % ethanol (V=200 µL) on an in-house made magnet. After an additional acetonitrile wash (V=200µL), 5 µL digestion solution (10 mM HEPES pH = 8.5 containing 0.5µg Trypsin (Sigma) and 0.5µg LysC (Wako)) was added to each sample and incubated overnight at 37°C. Peptides were desalted on a magnet using 2 x 200 µL acetonitrile and labelled with TMT (11 plex) on SP3 beads in 10 µL 100 mM HEPES pH=8.5 for 1h at 37°C. 0.4 mg TMT reaction reagent in acetonitrile was used per sample. The reaction was stopped using 0.5 % final concentration of hydroxylamine for 30 min at room temperature. Beads were pooled and peptides were eluted together using 5% DMSO. Next, we performed high pH offline peptide fractionation for mouse heart samples. TMT-labelled peptides were pooled to a total peptide amount of 50 µg. The sample was desalted using the StageTip technique, dried in a SpeedVac completely and resuspended in 10 µL of 10 mM ammonium hydroxide in 5% acetonitrile. The instrumentation consisted out of a ZirconiumTM Ultra HPLC and a PAL RTC autosampler system using the binary buffer system. AB) 10 mM ammonium hydroxide and 80% acetonitrile and 10 mM ammonium hydroxide. Peptides were separated according to their hydrophobicity using an in-house packed column (length = 40 cm, inner diameter = 175 µm, 2.7-µm beads, PoroShell, Agilent Technologies) column. The instrument was controlled using the software Chronos (Axel Semrau GmbH). The total gradient length was 40 min and in total 36 fractions were collected (1/30 s) and subsequently concentrated using a SpeedVac to complete dryness.

Liquid Chromatography and Mass Spectrometry for heart proteomics

Eluted peptides in each fraction were dissolved in 10 µL of 2% formic acid and 2% acetonitrile in LC-MS H2O. 3 µL were injected for each fraction. The instrumentation consisted of a nanoLC 1200 (Thermo Fisher) coupled via a nano-electrospray ionization source to a Qexactive HF-x mass spectrometer. Peptides were separated on a 20 cm in-house packed column (75 µm inner diameter, PoroShell, 2.7 µm beads) using a binary buffer system: AB) 0.1% formic acid and 0.1% formic acid in 80% acetonitrile. The gradient time was 25 min.

MS1 spectra were acquired using a mass range from 250 to 1650 Th, a resolution (at 200 m/z) of 60,000 and an AGC target of 3e6 allowing a maximum injection time of 20 ms using profile mode. MS2 spectra were acquired at a resolution of 45,000 using a maximum injection time of 96 ms. The AGC target was to 1e5. The isolation window was set to 0.8 m/z. The fixed first mass was set to 110 m/z. The spectra were acquired in centroid mode.

Analysis of proteomic data

Acquired mass spectra were subjected to MaxQuant (2.0.3.0) based analysis using the implemented Andromeda search engine. The input Fasta file contained reviewed Uniprot protein entries of the Mus musculus reference proteome (downloaded 12.2021, 17.029 protein entries). TMT MS2 Quantification was selected using the first channel (126) as a reference (all intensities values are normalized to this value by division) since it contained a pool protein digest from all samples. Oxidation at methionine residues and protein N-term acetylation were defined as variable modifications. The false discovery rate (FDR) was controlled to 1% at the protein and peptide-spectrum-match level using the implemented ‘revert’ algorithm. The mass tolerances were used as defined by default for FTMS instruments: 20 ppm at MS/MS level. The match between runs algorithm was enabled using default settings. A set of common
Metabolite extraction of polar and lipophilic metabolites from mouse heart tissues

For the extraction of total lipids, 15-20 mg of snap-frozen murine heart tissue was homogenized to a fine powder using a ball mill-type grinder (Tissue Lyser2: http://www.quiagen.com/) equipped with a 10-sample holder (Retsch). For the homogenization of the cell pellets one liquid nitrogen cooled 5 mm stainless steel balls was added to each tube and the frozen material was disintegrated for 1 min at 25 Hz. Polar metabolites and lipids were extracted by adding 1 mL of pre-cooled (-20°C) extraction buffer (methyl tert-butyl ether (MTBE); methanol: UPLC-grade water 5:3:2 [v:v:v]), containing 20 μL of EquiSplash Lipidomix (https://avantlipids.com/) as internal standard. The tubes were immediately vortexed until the sample was well re-suspended in the extraction buffer. The homogenized samples were then incubated on a cooled (4°C) orbital mixer at 1500 rpm for 30 min. After this step, the metal ball was removed and the samples were centrifuged for 10 min at 21,100 g in 4°C. The supernatant was transferred to a fresh tube and 100 μL of MTBE and 100 μL of UPLC-grade water were added to each. The tubes were immediately vortexed before incubating them for an additional 10 min on a cooled (15°C) orbital mixer at 1500 rpm. After this step, the samples were centrifuged for 10 min at 15°C and 16,000 x g, which provided a 2-phase separation of lipid and polar metabolites. The upper, MTBE phase, contains the lipids, while the lower, methanol-water, phase contains the polar and semi-polar metabolites. For the analysis of rapamycin, 600 μL of the upper lipid phase were collected into a fresh tube, which was stored at -80°C for the mass spectrometric analysis. The remaining lower (polar) phase (~800 μL) was immediately dried in a SpeedVac concentrator and stored dry at -80°C until mass spectrometric analysis.

Metabolite extraction of polar and lipophilic metabolites from MEFs

MEFs were plated 24 h before collecting. Growth media was removed from each well on the day of sample collection and the cells were washed twice, using 1 mL of 75 mM ammonium carbonate pH 7.4 (Sigma) wash buffer warmed to 37°C. MEFs were plated 24 h before collecting. Growth media was removed from each well on the day of sample collection and the cells were washed twice, using 1 mL of 75 mM ammonium carbonate pH 7.4 (Sigma) wash buffer warmed to 37°C. Cells were immediately scraped and the whole volume, including the precipitated cellular material, was transferred to a labelled 1.5 ml Eppendorf tube stored on ice. This extraction procedure from the plates was repeated another two times, leading to the collection of a total of 1.2 mL of metabolite extract from each sample.

Once the extracts were collected, they were centrifuged for 10 min at 4°C and 21,000 x g. The cleared supernatant was transferred to a fresh 1.5 ml Eppendorf tube and the polar metabolite extract was dried down immediately in a SpeedVac concentrator (ScanVac) set to 20°C and 1000 rpm until tubes were completely dried. These samples can then be used directly for analysis of they can be stored at -80°C until measured on the diverse LC-MS systems. The cellular pellet, which contains the precipitated protein, was used for protein quantification (BCA Protein Assay Kit) and later on these values were utilized for metabolite normalization.

Targeted liquid chromatography-high-resolution mass spectrometry-based (LC-HRS-MS) analysis of amine-containing metabolites from mouse heart tissue and MEF cell cultures

The LC-HRMS analysis of amine-containing compounds was performed using an adapted benzoylchlorid-based derivatization (Wong et al., 2016). In brief: The polar fraction of the metabolite extract was re-suspended in 300 μL of LC-MS-grade water (Optima-Grade, Thermo Fisher Scientific) and incubated at 4°C for 15 min on a thermomixer. The re-suspended extract was centrifuged for 5 min at 16,000 x g at 4°C and 50 μL of the cleared supernatant were mixed with 25 μL of 100 mM sodium carbonate (Sigma), followed by the addition of 25 μL 2% [v/v] benzoylchloride (Sigma) in acetonitrile (Optima-Grade, Thermo Fisher Scientific). Samples were vortexed and kept at 20°C until analysis. For the LC-HRMS analysis, 1 μL of the derivatized sample was injected onto a 100 x 2.1 mm HSS T3 UPLC column (Waters). The flow rate was set to 400 μl/min using a binary buffer system consisting of buffer A (10 mM ammonium formate (Sigma), 0.15% [v/v] formic acid (Sigma) in LC-MS-grade water (Optima-Grade, Thermo Fisher Scientific)). Buffer B consisted solely of acetonitrile (Optima-grade, Thermo Fisher-Scientific). The column temperature was set to 40°C, while the LC gradient was: 0% B at 0 min, 0-15% B 0–4.1 min; 15-17% B 4.1 – 4.5 min; 17-55% B 4.5-11 min; 55-70% B 11 – 11.5 min, 70-100% B 11.5 - 13 min; B 100% 13 - 14 min; 100-0% B 14 -14.1 min; 0% B 14.1-19 min; 0% B. The mass spectrometer (Q-Exactive Plus, Thermo Fisher Scientific) was operating in positive ionization mode recording the mass range m/z 100-1000. The heated ESI source settings of the mass spectrometer were: Spray voltage 3.5 kV, capillary temperature 300°C, sheath gas flow 60 AU, aux gas flow 20 AU at a temperature of 330°C and the sweep gas to 2 AU. The RF-lens was set to a value of 60.

Liquid Chromatography-High Resolution Mass Spectrometry-based (LC-HRMS) analysis of rapamycin

The stored (-80°C) lipid extracts were dried in a SpeedVac concentrator before analysis and lipid pellets were re-suspended in 200 μL of UPLC-grade acetonitrile: isopropanol (70:30 [v:v]). Samples were vortexed for 10 seconds and incubated for 10 min on a thermomixer at 4°C. Re-suspended samples were centrifuged for 5 min at 16,000 x g and 4°C, before transferring the cleared supernatant to 2 ml glass vials with 200 μl glass inserts (Chromatography Zubehör Trott, Germany). All samples were placed in an Acquity iClass UPLC (Waters) sample manager at 6°C. The UPLC was connected to a Tribrid Orbitrap HRMS, equipped with a heated ESI (HESI) source (ID-X, Thermo Fisher Scientific).
Of each lipid sample 1 µl was injected onto a 100 x 2.1 mm BEH C8 UPLC column, packed with 1.7 µm particles (Waters). The flow rate of the UPLC was set to 400 µl/min and the buffer system consisted of buffer A (10 mM ammonium acetate, 0.1% acetic acid in UPLC-grade water) and buffer B (10 mM ammonium acetate, 0.1% acetic acid in UPLC-grade acetonitrile/isopropanol 7:3 [v/v]). The UPLC gradient was as follows: 0-1 min 45% A, 1-4 min 45-25% A, 4-12 min 25-11% A, 12-15 min 11-1% A, 15-20 min 1% A, 20-20.1 min 1-45% A and 20.1-24 min re-equilibrating at 45% A. This leads to a total runtime of 24 min per sample.

The ID-X mass spectrometer was operating in positive ionization mode monitoring a mass range between m/z 150-1500. The resolution was set to 120,000, leading to approximately 4 scans per second. The RF lens was set to 60%, while the AGC target was set to 250%. The maximal ion time was set to 100 ms and the HESI source was operating with a spray voltage of 3.5 kV in positive ionization mode. The ion tube transfer capillary temperature was 300°C, the sheath gas flow 60 arbitrary units (AU), the auxiliary gas flow 20 AU and the sweep gas flow was set to 1 AU at 330°C.

Targeted data analysis of rapamycin was performed using the quan module of the TraceFinder 4.1 software (Thermo Fisher Scientific). Peak areas of [M + NH4]+ ion of rapamycin were extracted using a mass accuracy (<2 ppm) and a retention time tolerance of <0.05 min as compared to an independently measured rapamycin (LC Laboratories) standard.

**Anion-Exchange Chromatography Mass Spectrometry (AEX-MS) for the analysis of anionic metabolites from mouse tissue**

Extracted metabolites from murine heart tissue were re-suspended in 300 µl of Optima UPLC/MS grade water (Thermo Fisher Scientific). After 15 min incubation on an orbital mixer at 4°C and a 5 min centrifugation at 16,000 x g at 4°C, 100 µl of the cleared supernatant were transferred to polypropylene autosampler vials (Chromatography Accessories Trott, Germany).

The samples were analysed using a Dionex ion chromatography system (Integration, Thermo Fisher Scientific) as described previously (Schwaiger et al., 2017). In brief, 5 µl of polar metabolite extract were injected in full loop mode using an overfill factor of 1, onto a Dionex IonPac AS11-HC column (2 mm x 250 mm, 4 µm particle size, Thermo Fisher Scientific) equipped with a Dionex IonPac AG11-HC guard column (2 mm x 50 mm, 4 µm, Thermo Fisher Scientific). The column temperature was held at 30°C, while the auto sampler was set to 6°C. A potassium hydroxide gradient was generated using a potassium hydroxide cartridge (Eluent Generator, Thermo Scientific), which was supplied with deionized water. The metabolite separation was carried at a flow rate of 380 µl/min, applying the following gradient conditions: 0-3 min, 10 mM KOH; 3-12 min, 10–50 mM KOH; 12-19 min, 50-100 mM KOH, 19-21 min, 100 mM KOH, 21-22 min, 100-10 mM KOH. The column was re-equilibrated at 10 mAU for 8 min.

For the analysis of metabolite pool sizes the eluting compounds were detected in negative ion mode using full scan measurements in the mass range m/z 50–750 on a Q-Exactive HF high resolution MS (Thermo Fisher Scientific). The heated electrospray ionization (ESI) source settings of the mass spectrometer were: Spray voltage 3.2 kV, capillary temperature was set to 300°C, the sheath gas flow 60 arbitrary units (AU), the auxiliary gas flow 20 AU and the sweep gas flow was set to 1 AU at 330°C.

**Data analysis of LC-MS metabolites**

Semi-targeted data analysis for the samples was performed using the TraceFinder software (Version 4.1, Thermo Fisher Scientific). The identity of each compound was validated by authentic reference compounds, which were measured at the beginning and the end of the sequence.

**Cell culture, transfection and RNA interference**

SV40 immortalized and Cre-transduced monoclonal MEFs were maintained in DMEM-GlutaMAX (Gibco) containing 4.5 g l⁻¹ of glucose supplemented with 10% fetal bovine serum (Sigma). Cell lines were maintained at 37 °C and 5% CO₂ and were routinely tested for mycoplasma. Cell numbers were monitored by Trypan blue exclusion and cell counting using the Countess automated cell counter (Thermo Fisher Scientific). Cells were seeded at equal densities and grown to confluence over a period of 72 h without medium changes unless stated otherwise. For galactose treatment, MEFs were maintained in DMEM- without glucose (Gibco) supplemented with 4.5 g/l of galactose and with 10% dialyzed fetal bovine serum (Gibco) for 22 h. Opti-MEM + GlutaMAX (Gibco) and lipofectamine RNAiMax (Invitrogen) were used for reverse transfection of endoribonuclease-prepared short interfering RNA (siRNA; 50 nM). The siRNAs used for transfection are listed in STAR Methods. Cells were transfected 48 h before galactose treatment.

Cells were treated with 10 µM ferrostatin-1 (Fer-1) and media was refreshed every day. Cells were seeded every 72 h and collected after 7 days in total in culture with or without Fer-1. 1 µM QVD, 40 µM necrostatin-1 or 200 nM sodium selenite was added to media...
24 h prior to analysis. To assess ferroptosis, 40,000 MEFs were seeded per well in a 96-well plate and incubated overnight. Media was changed to fresh media containing 300 nM cell viability dye YOYO-1 (Invitrogen) with or without the following chemicals: 1 μM erastin, 10 μM ferrostatin-1, 1 μM QVD, 40 μM necrostatin-1, 1 μM ISRIB, and 200 nM sodium selenite. Cells were imaged for every 2 h in an IncuCyte S3 Live-Cell Analysis System (Essen Bioscience, Sartorius). For knock-down experiment cells were reverse-transfected as described above. Media was exchanged 24 h after transfection. Cells were trypsinized, counted and re-plated with new reverse-transfection after 72 h into 96-well plate for overnight culture prior to the ferroptosis assay.

**FACS analysis**

For MitoSOX experiment MEFs were washed with PBS and harvested with trypsinization. For positive control, we incubated cells with 10 μM antimycin A for 10 min in 37°C. Cells were treated with 5 μM mitoSOX in PBS at 37°C for 20 min, washed twice with PBS and stained with DAPI as a live/dead cell marker. Cells were filtered through 50 μm cell strainer and analyzed with BD FACS CANTOII with band pass 585/42 for laser 488 nm and 450/50 laser 405 nm and with FlowJo software (BD).

**Live-cell immunofluorescent analysis**

Mitochondrial iron content was determined according to guidelines of the manufacturer (Mito-Ferro Green kit, Dojinjo). Shortly; cells were plated on coverslips and incubated overnight. Cells were washed with Imaging Media and Ac-MIFluNox stain (5 μM) and MitoTracker Deep Red FM (Thermo Fisher Scientific) (0.5 μM) was added in Imaging Media (Gibco) to cells. Cells were incubated for 30 min at 37 °C and washed with Imaging Media. Cells were immediately imaged in Imaging Media with Leica SP8-X. Ten cells/genotype were imaged in one experiment to keep within comparable time frame for each cell and experiment was repeated three times.

**RNA sequencing and quantitative PCR with reverse transcription**

Heart tissue was homogenized with Precells 24 tissue homogenizer (Bertin Instruments) with 2 x 10 sec 6000 rpm keeping samples on ice in between the cycles. Total RNA was extracted from mouse heart tissue samples (mice age four weeks) using NucleoSpin RNA (Macherey-Nagel). RNA was first poly(A) selected using poly(T) oligo-attached magnetic beads and mRNA was purified and fragmented using divalent cations synthesis under elevated temperature. Random primer reverse transcription was done for RNA fragments before second-strand cDNA synthesis with DNA Polymerase I and Rnase H. After end repair and A-tailing, indexing adaptors were ligated. The products were then purified and amplified to create the final cDNA libraries. After library validation and quantification (Agilent tape station), equimolar amounts of library were pooled. The pool was quantified by using the Pqelab KAPA Library Quantification Kit and the Applied Biosystems 7900HT Sequence Detection System. The pool was sequenced with a paired-end 100-nucleotide protocol on an Illumina NovaSeq6000 sequencer. Raw reads were mapped to the mouse genome (ENSEMBL release 9) using Kallisto version 0.45.0. (Bray et al., 2016). Gene counts were quantified in the same step. Differential gene expression was performed using the AGSeq library version 0.8.0 in Python version 3.6.5. Protein interaction networks were generated and visualized in Python version 3.6.5 using the py2cytoscape package for Cytoscape version 3.7.1. For pathway analysis, we utilized all significantly changed genes (p<0.05) and Ingenuity Pathway analysis software.

For RT-PCR, cDNA was synthesized using the GoScript Reverse Transcription Mix (Promega). RNA concentrations were measured with Nanodrop ONE (Thermo Scientific). RT–qPCR was performed using PowerSYBR Green PCR Master Mix (Applied Biosystems) and QuantStudio 5 and QuantStudio 6 Flex analysers (Applied Biosystems) with QuantStudio Design&Analysis software (Applied Biosystems). For each independent sample, RT–qPCR was performed in technical duplicates. The primer sequences for mouse transcripts are listed in the Table S1. For NanoString analysis 50 ng of total RNA was hybridized with custom made probe set for ISR target genes 16 h in 65 °C, loaded to the sample cartridge and analysed with nCounter Sprint and nSolver program (NanoString).

**SDS–PAGE, Blue-native and immunoblot analysis**

Mouse heart tissue was homogenized with Precells 24 tissue homogenizer (Bertin Instruments) with 2 x 20 sec at 6000 rpm in protein lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM MgCl2, 1 mM DTT, 10% glycerol, 2% SDS, 1% Triton X100 containing protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (PhosSTOP, Roche). Protein concentration was determined with BCA (Pierce). For protein extraction, MEFs were washed with cold PBS and resuspended in ice-cold RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.05% sodium deoxycholate, 1 mM EDTA) containing protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (PhosSTOP, Roche). Samples were boiled prior to SDS–PAGE in 5°C for 5 min except in a case of OXPHOS subunit analysis. Total proteins from tissue or cells (25–50 μg) were separated using SDS–PAGE, followed by transfer to nitrocellulose membranes and immunoblotting with the antibodies listed in the key resources table. Western blot images were acquired with Intas ChemoStar ECL Imager HR 6.0 and ChemoStar TS software (Intas).

For blue-native electrophoresis analysis we isolated mitochondria from heart tissue in homogenization buffer (220 mM mannitol, 20 mM sucrose, 2 mM EGTA, 0.1% BSA, 20 mM HEPES-KOH, pH 7.4, and protease inhibitor cocktail (Roche)) homogenized with ten strokes using a glass Teflon homogenizer at 1000 rpm on ice. The homogenates were centrifuged at 1,000 x g for 10 min at 4°C and supernatant was collected. Homogenisation was repeated twice. Mitochondrial fraction was isolated with centrifugation at 8000 x g for 10 min at 4°C. Protein concentration was determined using a Bradford assay. Mitochondria were solubilized with 6 g/g digitonin.
and proteins were separated in 3-9% native gradient gels, transferred to PVDF membrane and immunoblotted with the antibodies listed in key resources table.

**Immunofluorescence and histological staining**
Heart samples were freshly frozen in liquid nitrogen cooled isopentane in O.C.T Compound Embedding Medium (TissueTek). Frozen sections (8 µm) were blocked with 5% horse serum for one prior to an incubation overnight at 4°C in 2% BSA with selected antibodies that are listed in the key resources table. Sections were washed three times with PBS before incubation with secondary antibodies (AlexaFluor (Invitrogen)) for 1 h in the dark, washed again three times with PBS, stained with 4,6-diamidino-2-phenylindole (DAPI) and mounted with ProlongH Gold. For TUNEL staining, frozen sections (8 µm) were stained according to the guidelines of the manufacturer (BrdU, abcam) with counterstaining for DAPI. Images were acquired using a Leica SP8-X laser-scanning confocal microscope equipped with an 63x oil HC PL APO CS2 objective (NA 1.4) Four images/animal were analyzed by MDA staining and the total area of MDA positive stain was determined using the Fiji software. Cells from four images/animal were calculated for TUNEL analysis (200-300 cells in total).

For COX staining, frozen sections (8 µm) were incubated in 0,05 M phosphate buffer containing 220 mM sucrose, 2,5 mM 3’3 diaminobenzidine, 1 mg/ml catalase, 0,5mg/ml cytochrome c for 12 min. Masson’s Trichrome (Abcam) was performed to frozen sections (8 µm) according to the guideline of the manufacturer. Sections for COX and Masson’s were dehydrated in ascending alcohols and in xylene and mounted with Cytoseal Xyl (Thermo Scientific). Images were acquired using Nicon Eclipse Ci with a CFI P-Achromat 4x/0.10 and CFI P-Fluor 20x/0.50 objectives.

**QUANTIFICATION AND STATISTICAL ANALYSIS**
Sample size was chosen according to our previous experience and common standards. Power analysis was used to predetermine sample size for mouse experiments. The sample size included at least three independent cell culture wells or mice where statistical evaluation was performed. For metabolomic analysis of anionic metabolites, we excluded one WT mouse sample and two Cox10 samples as clear outliers based on PC clustering blots. Experiments were repeated as detailed in the figure legends. Mice were assigned to experimental groups based on genotypes available. Analyses were not blinded because experiments were performed and analysed by the same researcher except for microscopy imaging where samples were numbered. The N number for all MEF cell experiments represent independent experimental cell cultures. Data analysis was performed with Prism GraphPad 9 and Instant Clue. Images were processed with ImageJ and schematics were created with Adobe Illustrator 26.0.2 and BioRender.com.