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Article

Keywords: Mitofusin 1 and 2 (Mfn1 and Mfn2), mtDNA content, glucose homeostasis, diabetes

Posted Date: April 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-310904/v1>

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Mitofusin 1 and 2 regulation of mtDNA content is a critical determinant of glucose homeostasis

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ABSTRACT

The dynamin-like GTPases Mitofusin 1 and 2 (Mfn1 and Mfn2) are essential for mitochondrial function, which has been principally attributed to their regulation of fission/fusion dynamics. Here, we report that Mfn1 and 2 are critical for glucose-stimulated insulin secretion (GSIS) primarily through control of mtDNA content. Whereas Mfn1 and Mfn2 individually were dispensable for glucose homeostasis, combined Mfn1/2 deletion in β -cells reduced mtDNA content, induced mitochondrial fragmentation, and impaired respiratory function, ultimately resulting in severe glucose intolerance. Importantly, gene dosage studies unexpectedly revealed that Mfn1/2 control of glucose homeostasis was dependent on maintenance of mtDNA content, rather than mitochondrial structure. Indeed, pharmacologic mitofusin agonists rescued islet mtDNA depletion due to mitofusin deficiency independent of changes on mitochondrial structure. Mfn1/2 maintain mtDNA content by regulating the expression of the crucial mitochondrial transcription factor Tfam, as Tfam overexpression ameliorated the reduction in mtDNA content and GSIS in Mfn1/2-deficient β -cells. Thus, the primary physiologic role of Mfn1 and 2 in β -cells is coupled to preservation of mtDNA content rather than mitochondrial architecture, and Mfn1 and 2 may be promising targets to overcome mitochondrial dysfunction and restore glucose control in diabetes.

INTRODUCTION

Mitochondrial dynamics, the balance of fusion and fission of mitochondrial networks, is essential to control mitochondrial structure. Mitochondrial fission and fusion are governed by several key proteins that maintain mitochondrial quality control (1, 2). Fission is primarily regulated by the GTPase dynamin-related protein 1 (Drp1), while fusion is controlled by both outer and inner mitochondrial membrane machinery. The dynamin-related large GTPase optic atrophy protein 1 (Opa1) controls inner membrane fusion, while outer membrane fusion is controlled by two GTPases known as Mitofusin 1 and 2 (Mfn1 and Mfn2). Mitofusin 1 and 2 share ~80% sequence similarity and contain homologous functional domains, suggesting they play both overlapping and unique roles in metabolic function largely ascribed to their control of mitochondrial structure (3).

Imbalances in mitochondrial dynamics in metabolically active tissues have been implicated in many human diseases, including neurodegenerative conditions, cancer, cardiovascular disease, and diabetes (2). Mitochondria provide the energy necessary for β -cell insulin release (4, 5), and abnormalities in mitochondrial structure and bioenergetics have been observed in the β -cells of humans with type 2 diabetes (T2D; (6, 7)). Thus, strategies to understand and overcome mitochondrial dysfunction in β -cells are of great appeal for the treatment of diabetes. However, the role of the core fission/fusion machinery in β -cells is less clear. Reduced β -cell Mfn2 expression has been observed secondary to the deposition of toxic islet amyloid polypeptide oligomers observed in T2D as well as in mouse models of T2D (8, 9), yet the functions of Mfn1 and Mfn2 in β -cells *in vivo* are unexplored.

Here we elucidate a key physiologic role for Mfn1 and 2 in the maintenance of glucose homeostasis that unexpectedly occurs through the preservation of β -cell mtDNA content, rather than mitochondrial structure. Utilizing several genetic mouse models, high resolution imaging, and transcriptomic profiling, we show that Mfn1 and 2 act in tandem to preserve β -cell mitochondrial health and, consequently, glucose-stimulated insulin secretion (GSIS). Mfn1 and 2 maintain mtDNA content by preventing loss of the mitochondrial transcription factor Tfam, which

is a master regulator of mtDNA copy number control and mtRNA expression (10, 11). Indeed, adenoviral overexpression of Tfam ameliorates reductions in mtDNA content and GSIS in Mfn1/2-deficient β -cells, illustrating the critical importance of mtDNA copy number control by Mfn1/2 to promote glucose homeostasis.

RESULTS

Combined deficiency of Mfn1 and Mfn2 in β -cells leads to severe glucose intolerance and reduced glucose-stimulated insulin secretion

Mitochondrial health is vital to support β -cell insulin secretory responses to glucose or other nutrient stimuli. We hypothesized that Mfn1 and Mfn2 are required for GSIS in β -cells through their regulation of mitochondrial function. To test this hypothesis, we generated mice bearing β -cell specific deletion of Mfn1 alone (*Mfn1*^{loxP/loxP}; *Ins1*-Cre), Mfn2 alone (*Mfn2*^{loxP/loxP}; *Ins1*-Cre), and combined deletion of both Mfn1 and 2 (*Mfn1*^{loxP/loxP} *Mfn2*^{loxP/loxP}; *Ins1*-Cre), hereafter known as β -Mfn1^{KO}, β -Mfn2^{KO}, and β -Mfn1/2^{DKO} mice, respectively. β -Mfn1^{KO}, β -Mfn2^{KO}, and β -Mfn1/2^{DKO} mice each exhibited an efficient reduction in Mfn1 and/or Mfn2 islet protein expression, respectively, when compared to littermate controls (Ctrl; Figures 1A-B). We did not observe a compensatory increase in Mfn2 expression in β -Mfn1^{KO} islets, nor in Mfn1 expression in β -Mfn2^{KO} islets (Figures 1A-B). Further, we did not observe differences in expression of the other core fission/fusion proteins, Drp1 and Opa1, following deletion of Mfn1 or Mfn2 (Figure S1A).

While loss of either Mfn1 or Mfn2 alone has been shown to impair glucose homeostasis in other metabolic tissues (3, 12), we were surprised to find that both β -Mfn1^{KO} and β -Mfn2^{KO} mice exhibited normal glucose homeostasis during an intraperitoneal glucose tolerance test (IPGTT; Figure 1C). We also observed no changes in insulin secretion in single β -Mfn1^{KO} or β -Mfn2^{KO} mice after glucose administration *in vivo* (Figure 1D) or in isolated islets (Figure 1E). However, combined deletion of both Mfn1 and Mfn2 resulted in severe glucose intolerance (Figure 1B; $p < 0.005$ by ANOVA vs. Ctrl), due to markedly reduced GSIS (Figures 1D-E). Reductions in GSIS in β -Mfn1/2^{DKO} mice were not secondary to changes in insulin content (Figure S1B).

To evaluate other etiologies of glucose intolerance in β -Mfn1/2^{DKO} mice, we first measured β -cell mass. We observed no significant differences in β -cell mass between genotypes (Figure 1F). There were also no differences in body weight or peripheral insulin sensitivity between the groups (Figures S1C-D). Taken together, these studies suggest that Mfn1 and 2 are individually

dispensable for glycemic control and GSIS, but together play vital complementary roles for the maintenance of glucose tolerance and β -cell insulin release.

Mfn1 and 2 regulate β -cell mitochondrial structure and respiratory function

Given the connections between mitochondrial function and dynamics, we asked whether mitochondrial respiration and architecture would be defective in the islets of β -Mfn1/2^{DKO} mice. Indeed, islets isolated from β -Mfn1/2^{DKO} mice displayed a reduced glucose-stimulated oxygen consumption rate (OCR; Figure 2A) when compared to Ctrl islets. We did not observe differences in glycolysis as measured by extracellular acidification rate (data not shown). Differences in glucose-stimulated OCR between Ctrl and β -Mfn1/2^{DKO} islets were no longer observed following exposure to the Complex III inhibitor antimycin A, suggesting that Mfn1/2-deficient islets primarily possess a defect in β -cell mitochondrial respiration (Figure 2A).

Loss of key mediators of mitochondrial fusion disrupt mitochondrial network balance and favor increased mitochondrial fission, often leading to the appearance of increased mitochondrial fragmentation (13). We thus examined mitochondrial structure in Mfn1- and/or Mfn2-deficient β -cells by two independent and complementary methods. First, we assessed β -cell mitochondrial ultrastructure by transmission electron microscopy (TEM). While β -cells from all groups had the expected appearance of electron dense insulin granules, β -cells from β -Mfn1/2^{DKO} mice had reductions in mitochondrial aspect ratio, form factor, and perimeter, and increased mitochondrial circularity (Figures 2B-C), indicating smaller, fragmented mitochondria. Secondly, we examined mitochondrial morphology and networks utilizing high-resolution deconvolution imaging of the mitochondrial marker succinate dehydrogenase A (SDHA) to generate three-dimensional (3D) reconstructions of β -cell mitochondria (14, 15). Consistent with our findings using TEM, the 3D mitochondrial appearance was similar between Ctrl and single β -Mfn1^{KO} or β -Mfn2^{KO} β -cells (Figure 2D). However, Mfn1/2-double deficient mice showed a dramatic increase in the frequency of smaller, vesiculated β -cell mitochondrial networks, consistent with fragmentation (Figure 2D).

3D quantification confirmed that measures of mitochondrial morphology (reduced mitochondrial volume and increased sphericity) and network/branch complexity (reduced branch number and branch junctions) were significantly impaired in β -Mfn1/2^{DKO} mice, commensurate with an increase in mitochondrial fragmentation due to decreased mitochondrial fusion (Figure 2E). Together, these data demonstrate that Mfn1 and 2 combine to maintain β -cell mitochondrial structure and function.

Mfn1 and 2 maintain β -cell mtDNA content

Mitochondrial DNA copy number is vital for β -cell function and glucose homeostasis (16, 17). Thus, we next examined whether mtDNA content was altered by loss of Mfn1 and/or Mfn2 in β -cells. We observed a small, yet significant, reduction in mtDNA content in islets of β -Mfn2^{KO} mice that was not observed in islets of β -Mfn1^{KO} mice (Figure 3A). Interestingly, mtDNA depletion substantially worsened in the islets of β -Mfn1/2^{DKO} mice (Figure 3A), suggesting that loss of Mfn1 exacerbates the depletion of mtDNA caused by Mfn2 deficiency. We next assessed expression of the 13 mitochondrially encoded genes and observed a consistent and significant reduction in mtRNA expression in β -Mfn2^{KO} islets that was exacerbated in β -Mfn1/2^{DKO} mice (Figure 3B). Concordantly, examination of protein expression of mitochondrial OXPHOS subunits by Western blot also revealed a reduction of the mitochondrially encoded subunit of Complex IV, mt-Co1, in β -Mfn2^{KO} islets and β -Mfn1/2^{DKO} islets (Figures 3C-D). We also observed significant reductions of nuclear encoded subunits of Complex I (Ndufb8) in both β -Mfn2^{KO} and β -Mfn1/2^{DKO} islets and Complex III (Uqcrc2) in β -Mfn1/2^{DKO} islets, but no significant depletion of nuclear encoded Complex II (Sdhb) and Complex V (Atp5a) (Figures 3C-D). These studies reveal that both Mfn1 and 2 (and Mfn2 alone to a lesser degree) are necessary to maintain mtDNA copy number, mtRNA expression, and expression of OXPHOS subunits in β -cells.

Defects in β -cell mitochondrial fusion induce glucose intolerance upon loss of mtDNA content

Our observations showing distinct and overlapping contributions of *Mfn1* and 2 in the regulation of β -cell mitochondrial function led us to hypothesize that loss of mitochondrial fusion would lead to glucose intolerance due to mtDNA depletion. However, approaches to clarify the specific contribution of mtDNA depletion to metabolic dysfunction following imbalances in mitochondrial dynamics have been elusive. To this end, we generated animals harboring only a single functional allele of either *Mfn1* (β -*Mfn1*^{+/-}*Mfn2*^{KO}) or *Mfn2* (β -*Mfn1*^{KO}*Mfn2*^{+/-}) to ascertain the relative importance of mtDNA content and structure in the maintenance of glycemic control. For robustness of interpretation, these experiments were performed simultaneously alongside littermate controls and β -*Mfn1/2*^{DKO} mice as previously presented (Figures 1-3). As expected, neither β -*Mfn1*^{+/-}*Mfn2*^{KO} nor β -*Mfn1*^{KO}*Mfn2*^{+/-} mice developed changes in β -cell mass, body weight, or insulin sensitivity (Figures S2A-C). Interestingly, 3D quantification of mitochondrial morphology and network integrity revealed that, similar to β -*Mfn1/2*^{DKO} mice, a single allele of either *Mfn1* or *Mfn2* was not sufficient to maintain β -cell mitochondrial structure and led to mitochondrial fragmentation (Figure 4A-B).

Despite mitochondrial fragmentation in both β -*Mfn1*^{+/-}*Mfn2*^{KO} and β -*Mfn1*^{KO}*Mfn2*^{+/-} mice, *only* β -*Mfn1*^{+/-}*Mfn2*^{KO} mice developed glucose intolerance and impaired GSIS similar to that of β -*Mfn1/2*^{DKO} mice (Figures 4C-D). We also found significant reductions in mtDNA content associated with the loss of GSIS observed in β -*Mfn1*^{+/-}*Mfn2*^{KO} and β -*Mfn1/2*^{DKO} mice (Figure 4E). Thus, maintenance of a single functional allele of *Mfn2*, but not *Mfn1*, is able to preserve glucose homeostasis, mtDNA content, and insulin secretion, despite being insufficient to maintain β -cell mitochondrial network integrity. Importantly, these results disjoin β -cell function from changes in mitochondrial morphology, and implicate mtDNA copy number as the vital purveyor of glycemic control following loss of mitochondrial fusion.

Mitofusin agonists restore mtDNA content independent of changes in mitochondrial structure

We next asked if pharmacologically targeting mitofusins could restore mtDNA levels in models of mitofusin deficiency independent of changes in mitochondrial structure. To this end, we employed recently described tandem mitofusin agonists, (2-{2-[(5-cyclopropyl-4-phenyl-4H-1,2,4-triazol-3-yl)sulfanyl]propanamido}-4H,5H,6H-cyclopenta[b]thiophene-3-carboxamide and 1-[2-(benzylsulfanyl)ethyl]-3-(2-methylcyclohexyl)urea (hereafter called Mfn agonists), which together mimic an open Mfn2 conformation favoring mitochondrial fusion but require some residual mitofusin levels to be effective (18). We initially treated control and β -Mfn2^{KO} islets, which exhibit reduced mtDNA content without changes in mitochondrial structure (Figures 3A and 2B-E). To avoid systemic effects of modifying mitochondrial fission/fusion balance in other tissues (1), we utilized Mfn agonists on isolated islets. Interestingly, Mfn agonists restored mtDNA content to baseline in β -Mfn2^{KO} islets (Figure 5A).

We next evaluated the effects of Mfn agonists in other models of mitofusin deficiency, as Mfn2 expression is reduced in the islets of models of T2D (9). We assessed the expression of Mfn1 and 2 in islets isolated from the leptin receptor deficient *db/db* mouse model, which develops obesity, progressive β -cell mitochondrial and insulin secretory dysfunction, and eventual β -cell failure (19-23). We observed that Mfn2, but not Mfn1, protein levels were reduced in islets of 10-12 week old *db/db* mice when compared to *db/+* controls (Figure 5B), measured at an age prior to β -cell failure (19, 20). Similar to observations in β -Mfn2^{KO} islets, *db/db* islets exhibited reduced mtDNA content without alterations in mitochondrial structure (Figures 5C-D). Concordantly, treatment with Mfn agonists also rescued mtDNA levels in *db/db* islets, while leaving mitochondrial structure unchanged (Figures 5C-D). We also observed that Mfn agonists modestly improved GSIS in the islets of *db/db* mice (Figure 5E). Taken together, these results indicate that Mfn agonists restore mtDNA content independent of changes in mitochondrial structure. Moreover,

these results extend the association between mitofusin deficiency, mtDNA depletion, and impaired GSIS in β -cells.

Mfn1 and 2 deficiency induces a transcriptional signature consistent with defects in mitochondrial metabolism and alterations in mtDNA replication

To begin to understand the mechanisms underlying control of mtDNA content by Mfn1 and 2, we analyzed bulk RNA sequencing (RNAseq) data generated from islets of β -Mfn1^{KO}, β -Mfn2^{KO}, and β -Mfn1/2^{DKO} mice as well as littermate controls. Initial hierarchical clustering analyses based on the top 500 most highly expressed genes revealed that only β -Mfn1/2^{DKO} islets clustered by genotype, while we did not observe discrete clustering across Ctrl or single β -Mfn1^{KO} and β -Mfn2^{KO} islets due to very few differences in expression between these groups (Figures 6A and S3A-B). Gene ontology and pathway analyses of differentially expressed genes from β -Mfn1/2^{DKO} islets revealed significant changes related to endocrine hormone/insulin secretion, β -cell signature genes, as well as several metabolic pathways (Figures 6B and S3C-D), consistent with the defects in β -cell function and mitochondrial metabolism observed in β -Mfn1/2^{DKO} mice.

The nearly 1,000 significantly dysregulated genes in β -Mfn1/2^{DKO} islets included downregulation of mitochondrial encoded transcripts, as well as key β -cell signature genes such as Glut2 and MafA (Figures 6C and 6E). We also observed increases in expression of regulators of triglyceride metabolism, including Angptl4 and Angptl8 (24), the glycolytic/gluconeogenic enzyme AldoB, which is highly upregulated in models of β -cell mitochondrial dysfunction and in T2D islets (25, 26), as well as Nrf1, a key regulator of mitochondrial biogenesis (Figures 6C, 6E, and S3D; (27)). To resolve additional mitochondrial specific effectors that were differentially regulated following the loss of Mfn1 and 2, we next overlaid our RNAseq data on MitoCarta 2.0 (28), which contains a compendium of targets with strong evidence for localization to the mitochondria (Figure 6D). This revealed increased expression of several genes related to mtDNA replication machinery or associated with the mitochondrial nucleoid (Figures 6D-E), including the

critical mtDNA topoisomerase Top1mt (29), the mitochondrial co-chaperone Dnaja3/Tid1, which maintains mtDNA integrity (30), and the mitochondrial pyrimidine transporter Slc25a33, which is essential for mtDNA replication (31). We also observed upregulation in the mitochondrial AAA+ protease LonP1, which interacts with and regulates the stability of numerous proteins at the mitochondrial nucleoid (Figures 6D-E; (32-34)). Importantly, we did not observe changes in expression of other fission/fusion genes or regulators of mtRNA transcription, mtDNA repair, sirtuins, or mitophagy (Figure S3D). Taken together, these data indicate that Mfn1/2-deficiency induced a transcriptional signature consistent with an activation of mtDNA replication to potentially compensate for β -cell mtDNA depletion.

Mfn1 and 2 act through Tfam to regulate β -cell mtDNA copy number

The mechanisms underlying regulation of mtDNA by mitochondrial fusion have been controversial, and previous studies implicate several pathways that could reduce mtDNA content, including genome instability/increased mtDNA mutations, enhanced mitophagy, or impaired mtDNA replication (35-37). As our studies were completed in young mice, which bear infrequent mtDNA mutations (38), and we did not observe differences in expression of mtDNA repair enzymes (Figure S3D), we focused on assessments of mitophagy and mtDNA replication to better resolve the significant reduction of mtDNA observed in β -Mfn1/2^{DKO} islets. We first assessed rates of mitophagy following incubation of control and β -Mfn1/2^{DKO} islets with the cell permeable Mitophagy dye, and did not observe differences in mitophagy between genotypes (Figure S3E). We also did not observe a difference in frequency of autophagosomes bearing mitochondria by TEM, or changes in essential genes in the mitophagy pathway between control and β -Mfn1/2^{DKO} islets (Figure S3D and data not shown).

To assess regulators of β -cell mtDNA replication, we examined expression of the replisome proteins Ssbp1, Twinkle, and Polg, as well as Tfam, a nucleoid protein which both regulates mtDNA content and whose levels are proportional to mtDNA copy number (10). While

replisome proteins were largely unchanged, we observed a significant reduction in Tfam protein in β -Mfn1/2^{DKO} islets (Figures 7A-B). Tfam mRNA expression was unchanged in β -Mfn1/2^{DKO} mice (Figure S3D), suggesting the reduction in Tfam protein occurred post-transcriptionally. We further observed a significant increase in the level of LonP1 protein, which has been shown to lead to Tfam protein turnover (39, 40). We additionally confirmed reductions in Tfam and increases in LonP1 protein in β -Mfn1^{+/-}Mfn2^{KO} islets (Figure S4), correlating with the mtDNA depletion, glucose intolerance, and impaired GSIS observed in these mice (Figures 4C-E). In contrast, β -Mfn1^{KO}Mfn2^{+/-} mice, which develop mitochondrial fragmentation without glucose intolerance or mtDNA depletion, did not exhibit changes in Tfam and LonP1 (Figure S4 and Figures 4C-E). These results suggest that changes in Tfam and LonP1 expression following Mfn1/2 deficiency were not due to mitochondrial fragmentation.

To test for defects in mtDNA replication, we next assessed initiation of first strand replication. First strand synthesis in mitochondrial replication begins in the D-loop. However, a single stranded (ss) molecule that forms the classic D-loop structure called 7S, which is a non-replicative structure, shares this sequence. It is the elongation of the 7S sequence into the nearby region (Cytb) that demonstrates committed initiation of replication. Here we assessed the levels of ss and dsDNA at specific sequences by quantitative PCR to overcome expected sample limitations from studies with mouse pancreatic islets. Notably, import of nascent Tfam has been shown to increase the generation of 7S DNA due to its transcription initiation activity (41). We found a trend towards reduced total ss 7S sequences normalized to ds mtDNA in β -Mfn1/2^{DKO} islets (Figure 7C). However, the rate of 7S sequence extension into committed first strand replication was increased in β -Mfn1/2^{DKO} islets (Figure 7C), which could represent a compensatory response to mtDNA depletion.

Increased first strand replication intermediates heighten the levels of displaced second strand template, which is bound by the single strand binding protein Ssbp1 (42). Consistent with ss qPCR studies, in situ levels of Ssbp1 at the mtDNA nucleoid (detected by perinuclear anti-DNA

antibody staining), were significantly elevated in Mfn1/2-deficient β -cells (Figures 7D-E). The increase in Ssbp1 localization to mitochondrial nucleoids could represent binding to a higher quantity of single-stranded mtDNA replication intermediates and could also be consistent with observations of impaired completion of mtDNA replication previously reported following Mfn1/2-deficiency (36, 43). We also observed Ssbp1 nuclear localization (Figures 7D and F), which has been previously observed following mitochondrial stress (44). Further, we detected an increase in the volume of mtDNA nucleoid structures in Mfn1/2-deficient β -cells by high-resolution 3D imaging (Figures 7G-H), which are similar to previous reports of Tfam-deficiency but could also represent impaired nucleoid distribution (36, 45, 46). Taken together, our observations of reduced mtDNA content, mtRNA expression, as well as changes in single-stranded mtDNA products, Ssbp1 localization, and nucleoid size following Mfn1/2-deficiency could be suggestive of a role for Tfam in mediating the effects of Mfn1/2 in β -cell function.

To test whether increasing mtDNA copy number was sufficient to improve β -cell function in fusion-deficient islets, we overexpressed Tfam in β -Mfn1/2^{DKO} and littermate control islets. We transduced intact islets using adenoviral vectors encoding the human form of TFAM, thus allowing us to track overexpression of TFAM using human-specific TFAM anti-sera (Figures 8A-B and S5A). Transduction efficiency in β -cells was estimated at ~50% and was similar in both genotypes (Figure S5A and data not shown). As expected, we did not observe a rescue of mitochondrial structural defects in Mfn1/2-deficient β -cells by TFAM overexpression (data not shown). Despite limitations in transduction efficiency and expression, TFAM overexpression significantly ameliorated the reductions in both GSIS and mtDNA copy number in β -Mfn1/2^{DKO} islets (Figures 8C-D and S5B). Therefore, Mfn1 and 2 direct β -cell function, at least in part, through the regulation of Tfam-mediated mtDNA copy number control.

DISCUSSION

Here, we identify that Mfn1 and 2 promote mitochondrial fitness to fuel glucose-stimulated insulin release in pancreatic β -cells and maintain glucose homeostasis. Mfn1 or Mfn2 are individually dispensable for β -cell function, but together coordinately control mitochondrial respiration, structure, and mtDNA content. Mfn1 and 2 were thought to regulate cellular metabolism principally through the control of mitochondrial architecture. Our single allele mouse models directly demonstrate that Mfn1/2-deficiency is coupled to glucose intolerance through mtDNA loss rather than mitochondrial structure. Further, we observe that increased mtDNA copy number following Tfam overexpression is capable of ameliorating impaired GSIS in fusion-deficient β -cells. Thus, our studies position the maintenance of mtDNA content as the principal task necessary for Mfn1 and 2 to regulate β -cell function.

Our description of a central role for Mfn1 and 2 in β -cells through mtDNA content rather than mitochondrial structure elucidates a previously unappreciated role for mitochondrial fusion on cellular metabolic function. Mfn1 and 2 act in a complementary manner to support mitochondrial metabolism from development to adulthood. Both germline and cardiomyocyte-specific deletion of Mfn1 and 2 are associated with developmental or early postnatal demise (47, 48), presumed to be primarily caused by defects in mitochondrial network dynamics. Later descriptions of reduced mtDNA levels in constitutive or inducible loss of Mfn1/2 in skeletal muscle did not discern the physiologic importance of mtDNA depletion versus structure following fusion-deficiency (35, 49). Our results clarify a primary physiologic impact of mtDNA depletion rather than mitochondrial structure following Mfn1/2 deficiency in β -cells that may also underlie the physiologic importance of Mfn1 and 2 in other metabolic tissues. To our knowledge, this is the first time that these functions of Mfn1/2 have been separated in a physiological context.

While our data suggest that Tfam mediates the effects of Mfn1/2 on mtDNA content and GSIS in β -cells, the mechanisms for post-transcriptional loss of Tfam in β -Mfn1/2^{DKO} islets are unclear. Loss of Tfam may be due to increased LonP1 expression, which is known to degrade

Tfam (39, 40). Increases in LonP1 expression have also been reported to lead to the instability of nuclear-encoded subunits of Complex I and III, similar to our observations following Mfn1/2-deficiency (Figure 3D; (50)), which could further contribute to mitochondrial dysfunction. However, the function and transcriptional regulation of LonP1 in β -cells are unknown and require future study.

We observe that β -Mfn1/2^{DKO} islets attempt unsuccessfully to compensate for mtDNA depletion. Our analyses of first strand replication suggest that Mfn1/2-deficient β -cells increase the activation of early steps in mtDNA replication, which is supported by increased Ssbp1 localization to nucleoids to potentially bind newly formed ss-mtDNA. Despite these changes, mtDNA content remains low in Mfn1/2-deficient β -cells, suggesting that the increased commitment to replication is insufficient. Indeed, increased nucleoid Ssbp1 may be engaged in binding abnormal single-stranded replication products due to fusion and replication defects (36, 43). The increased expression of Top1mt found in Mfn1/2-deficient β -cells could also represent a response to increased mitochondrial replication stress (51). As Mfn2 is frequently observed at mitochondrial-ER contacts (MERCs; (52)), which are the sites of mtDNA replication (53), alterations in MERCs in β -Mfn1/2^{DKO} islets could possibly lead to impaired mtDNA replication. However, a recent report found that inducible loss of Mfn1/2 in adult β -cells did not alter MERC-dependent ER calcium release (54), and we did not observe ER dilation or overt changes in mitochondrial-ER proximity on transmission EM in β -Mfn1/2^{DKO} islets (Fig. 2B and data not shown), suggesting Mfn1/2 do not substantially affect β -cell MERCs. Additional experimentation will be required to dissect the molecular details underlying the connections between mitochondrial fusion and control of β -cell mtDNA replication.

Mitochondrial fission/fusion disturbances may lead to downstream extramitochondrial effects that could contribute to β -cell failure, but these have not yet been explored. Notably, β -cell Drp1 deficiency reduces second phase insulin release and the amplification pathway of insulin secretion (55), and β -cell Opa1 deficiency impairs entry into the cell cycle, thereby reducing β -

cell replication (56). We detect an increase of nuclear Ssbp1 following loss of mitochondrial fusion, which has been suggested to activate a mitochondrial stress response (44). We further observe significant hyperglycemia, reduced Tfam expression, and mtDNA depletion in young β -Mfn1/2^{DKO} mice that could potentially promote eventual glucotoxicity or an age-related loss of β -cell mass, similar to a previous report of selective Tfam deficiency in β -cells (17). Indeed, an independent group found that, similar to the GSIS defects we observe following constitutive loss of Mfn1/2 in β -cells, inducible Mfn1/2-deficiency in adult β -cells also impairs GSIS, leading to an eventual decline of β -cell mass with age (54). Investigation of the extramitochondrial effects which emanate from mtDNA depletion and disruptions in mitochondrial networking will be essential to understand how the early defects in mitochondria-potentiated insulin release cause progressive loss of β -cell mass.

Mitochondrial structural defects and age-related mtDNA depletion combine to impair β -cell function to increase T2D risk (6, 7, 57-59), thus, improving mitochondrial function would have significant translational benefits to restore β -cell health in diabetes. Our studies demonstrate the feasibility of targeting mtDNA copy number to improve GSIS in the setting of mitofusin deficiency. Further, recently identified compounds targeting pyrimidine metabolism to induce Mfn1/2 transcription (60) will be of interest to determine if they can improve β -cell function and mtDNA content. Our observation of the upregulation of mitochondrial pyrimidine transporter Slc25a33 in β -Mfn1/2^{DKO} islets is consistent with links between pyrimidine metabolism and mitochondrial fusion, which suggest these compounds could hold promise to enhance β -cell mitochondrial function. Future studies will be required to fully characterize the efficacy of overcoming mitochondrial defects *in vivo* to treat diabetes.

METHODS

Animals

Mfn1^{loxP/loxP} and *Mfn2*^{loxP/loxP} mice possessing loxP sites flanking exon 4 of the *Mfn1* gene and exon 6 of the *Mfn2* gene, respectively, were purchased from Jackson Laboratories (61). *Mfn1*^{loxP} and *Mfn2*^{loxP} mice were mated to *Ins1*-Cre (62) to generate experimental groups. *Ins1*-Cre-alone, *Mfn1*^{loxP/loxP} and *Mfn2*^{loxP/loxP} mice were phenotypically indistinguishable from each other and combined as controls (Ctrl). *Ins1*-Cre-alone were also phenotypically indistinguishable from wild-type C57BL/6N controls, consistent with previous reports from our group and others (data not shown; (14, 62)). All animals were all maintained on the C57BL/6N background. *db/+* and *db/db* mice, maintained on the BKS background, were purchased from Jackson Laboratories. Animals were housed on a standard 12-hour light/12-hour dark cycle with ad libitum access to food and water. All studies and endpoints in all figures were completed using both male and female mice, and results from both sexes were combined in all experimental groups.

Mitochondrial respirometry

Islet respirometry was measured using an XF96 extracellular flux analyzer (Seahorse Bioscience) according to manufacturer's instructions. Briefly, 20 similarly sized islets were plated per well of a Seahorse spheroid plate pre-treated with CellTak (Corning), similar to previously published approaches (63). Prior to respirometry profiling, islets were incubated for 1 hour in an atmospheric CO₂ incubator at 37°C and supplemented with mouse islet culture media (64, 65) comprised of pH 7.4 unbuffered RPMI1640 media (Seahorse Bioscience) and 2mM glucose prior to flux analyzer assays. Analysis was performed at baseline, upon exposure to 20mM glucose, and again after treatment with 2 μM antimycin A. OCR measurements were normalized to islet number confirmed by light microscopy (Zeiss Stemi 2000) after the completion of respirometry assays.

Transmission electron microscopy

Mouse islets were pelleted, fixed in 3% glutaraldehyde and 3% paraformaldehyde in 0.1 M Cacodylate buffer (CB; pH 7.2) overnight at 4°C. Islets were then embedded in 2% agarose as

described previously (66). Samples were then subjected to osmification in 1.5% $K_4Fe(CN)_6$ + 2% OsO_4 in 0.1 CB for 1 h, dehydrated by serial washes in EtOH (30%, 50%, 70%, 80%, 90%, 95% and 100%) and embedded in Spurr's resin by polymerization at 60° C for 24h. Polymerized resins were then sectioned at 90nm thickness using a Leica EM UC7 ultramicrotome and imaged at 70kV using a JEOL 1400 TEM equipped with an AMT CMOS imaging system. Mitochondrial structures (Aspect Ratio, Perimeter, Circularity) were analyzed and quantified by ImageJ. Form factor assessments were analyzed by ImageJ and quantified as described (15).

Mitochondrial morphology and subcellular localization

Mitochondrial morphology and subcellular localization analyses were performed on immunostained paraffin-embedded mouse pancreas tissue sections and dissociated islet cells as previously described (14). Z-stack images of immunostained pancreatic sections were captured with an IX81 microscope (Olympus) using an ORCA Flash4 CMOS digital camera (Hamamatsu) and subjected to deconvolution (CellSens; Olympus). Mitochondrial morphology and nucleoids was visualized using 3D-renderings generated with Imaris® imaging software (Bitplane). Quantitative 3D assessments of mitochondrial morphology and network were performed on ImageJ using MitoAnalyzer plugin (15). Co-localization analyses were performed on Z-stack images of immunostained dissociated islet cells using the Coloc2 plugin on ImageJ. Changes in relative nucleoid size were quantified from 3D deconvolution images of immunostained dissociated islet cells, detected by perinuclear anti-DNA antibody staining, using ImageJ.

mtDNA content and replication assays

Relative mtDNA content by qPCR with SYBR-based detection (Universal SYBR Green Supermix; Biorad) was conducted as previously described (67) following DNA isolation with the Blood/Tissue DNeasy kit (Qiagen) according to manufacturer's protocols. First strand mtDNA replication levels were measured by qPCR following digestion by Mnl I, which site specifically digest double stranded DNA (68). Briefly, primers specific to 7S mtDNA (5'-ACTCTTCTCTTCCATATGACTATCCC-3' and 5'-GGCCCTGAAGTAAGAACCAGATGT-3'),

whose amplicon contains a Mnl I site, were used to quantify relative single-stranded mtDNA forming the D-loop. Single-stranded DNA generated from the extension of 7S region was measured using primers specific to CYTB (5'-TGCATACGCCATTCTACGCTCA-3' and 5'-GTGATTGGGCGGAATATTAGGCTTC-3'), which also contains an Mnl I site. Primers specific to COXI (5'-GCAGGAGCATCAGTAGACCTAACA-3' and 5'-GCGGCTAGCACTGGTAGTGATAAT-3'), whose amplicon does not contain an Mnl I site, were used to quantify non-replicating double stranded mtDNA. The ratio of 7S or CYTB amplicons to the COXI amplicon were then calculated after qPCR of Mnl I digested DNA to determine the relative amount of single stranded mtDNA at the D-loop strand or initiation of first strand replication, respectively.

RNA sequencing, differential expression, and functional analysis

Sequencing was performed by the Advanced Genomics Core at University of Michigan Medical School. Total RNA was isolated and DNase treated using commercially available kits (Omega Biotek and Ambion, respectively). Libraries were constructed and subsequently subjected to 151bp paired-end cycles on the NovaSeq-6000 platform (Illumina). FastQC (v0.11.8) was used to ensure the quality of data. Reads were mapped to the reference genome GRCm38 (ENSEMBL), using STAR (v2.6.1b) and assigned count estimates to genes with RSEM (v1.3.1). Alignment options followed ENCODE standards for RNA-seq. FastQC was used in an additional post-alignment step to ensure that only high-quality data were used for expression quantitation and differential expression. Data were pre-filtered to remove genes with 0 counts in all samples. Differential gene expression analysis was performed using DESeq2, using a negative binomial generalized linear model (thresholds: linear fold change >1.5 or <-1.5, Benjamini-Hochberg FDR (P_{adj}) <0.05). Plots were generated using variations of DESeq2 plotting functions and other packages with R version 3.3.3. Genes were annotated with NCBI Entrez GeneIDs and text descriptions. Functional analysis, including candidate pathways activated or inhibited in comparison(s) and GO-term enrichments, was performed using iPathway Guide (Advaita). Primary data deposition at GEO is currently in process.

Adenoviral transfection

Isolated mouse islets were distended with Accutase for 1 min at 37° C as described previously (69). Islets were then transduced with 0.15 MOI of Ad.EV (expressing an empty vector) or Ad.hTFAM (expressing human-specific TFAM) for 48 hrs (viral particles purchased from Vector Biolabs). β -cell transduction was evaluated on dissociated islet cells by immunostaining with Pdx1 and human-specific TFAM anti-sera.

Flow cytometry

Following isolation and culture, live mouse islets were exposed to 100 nM Mtpagy dye (Dojindo Molecular Technologies) for 3 hours to assess time-dependent accumulation of mitochondria to acidic organelles by the relative fluorescence intensity of the dye per cell as described (70, 71). Islets were then dissociated into single cells, stained with DAPI (Thermo Fisher Scientific) and FluoZin-3 (Thermo Fisher Scientific), and resuspended in phenol red–free islet culture medium as previously described (14). Samples were analyzed on an LSR Fortessa flow cytometer (BD Biosciences). Single cells were gated using forward scatter and side scatter (FSC and SSC, respectively) plots, DAPI staining was used to exclude dead cells, and FluoZin-3 was used to identify β -cells as previously described (14, 72). Mtpagy measurements in β -cells were made using 488 nm excitation laser with a 710 nm emission filter and analyzed using FlowJo (Tree Star Inc.). A total of 5,000 β -cells was quantified from each independent islet preparation.

Glucose and insulin measurements

IPGTT, ITT and serum insulin measurements were performed as described previously (64, 65). Static incubation assays were performed on isolated mouse islets for insulin release as previously described (64, 65).

Islet isolation and cell culture

Mouse islets were isolated and cultured as previously described (64, 65). Cell treatments included DMSO (Fisher) and antimycin A (Sigma). Mitofusin agonist treatment included an equimolar mixture of two previously described compounds (0.5 μ M each; (18)), which were administered for

24 hours (2-{2-[(5-cyclopropyl-4-phenyl-4H-1,2,4-triazol-3-yl)sulfanyl]propanamido}-4H,5H,6H-cyclopenta[b]thiophene-3-carboxamide, CAS# 920868-45-7 and 1-[2-(benzylsulfanyl)ethyl]-3-(2-methylcyclohexyl)urea, CAS# 1007818-44-1; Enamine Ltd.).

Western blotting, quantitative PCR, and immunostaining

All assays were performed as previously described (14, 64, 65). β -cell mass was quantified as previously described (65). Antibodies used for Western blotting and immunostaining are listed in Supplementary Table 1.

Statistics

In all figures, data are presented as means $\pm$ SEM, and error bars denote SEM. Statistical comparisons were performed using unpaired two-tailed Student's t-tests or one-way ANOVA, followed by Tukey's post-hoc test for multiple comparisons, as appropriate (Prism GraphPad). A *P* value < 0.05 was considered significant.

Study approval

Animal studies were approved by the University of Michigan Institutional Animal Care and Use Committee.

Author Contributions

V.S. conceived, designed and performed experiments, interpreted results, drafted and reviewed the manuscript. J.Z. conceived, designed and performed experiments, interpreted results, and reviewed the manuscript. G.L.P. and E.C.R. designed and performed experiments and interpreted results. B.A.K. designed studies, interpreted results and reviewed the manuscript. S.A.S. conceived and designed the studies, interpreted results, drafted, edited, and reviewed the manuscript.

Acknowledgements

S.A.S was supported by the JDRF (CDA-2016-189, SRA-2018-539, COE-2019-861), the NIH (R01 DK108921, U01 DK127747), the Department of Veterans Affairs (I01 BX004444), the Brehm family, and the Anthony family. G.L.P. was supported by the American Diabetes Association (19-PDF-063). B.A.K. was supported by the Department of Veterans Affairs (I01 BX004444). The JDRF Career Development Award to S.A.S. is partly supported by the Danish Diabetes Academy and the Novo Nordisk Foundation. We acknowledge the Microscopy, Imaging and Cellular Physiology Core of the University of Michigan DRC (P30 DK020572) for assistance with imaging studies. We thank the University of Michigan Flow Cytometry Core for assistance with flow cytometry studies. Next generation sequencing was carried out in the Advanced Genomics Core at the University of Michigan. We acknowledge support from the Bioinformatics Core of the University of Michigan's Biomedical Research Core Facilities. We thank Drs. K. Claiborn, E. Walker, C. Rutledge, N. Desai, and members of the Soleimanpour laboratory for helpful advice.

Conflict of interest statement

The authors have declared that no conflict of interest exists.

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Figures

Figure 1

Loss of both Mfn1 and Mfn2 in β -cells impairs glucose tolerance and glucose-stimulated insulin release. (A) Expression of Mfn1 and Mfn2 by Western blot (WB) in islets isolated from 8-10-week-old Ctrl, β -Mfn1^{KO}, β -Mfn2^{KO} and β -Mfn1/2^{DKO} mice. Cyclophilin B serves as a loading control. Representative of 4 independent mice/group. (B) Densitometry (normalized to cyclophilin B) from studies in Figure 1A. n=4/group; *p<0.05 by ANOVA. (C) Blood glucose concentrations measured during IPGTT of 8-week-old Ctrl, β -Mfn1^{KO}, β -Mfn2^{KO} and β -Mfn1/2^{DKO} littermates. n=6-28/group. (p<0.005 by ANOVA for β -Mfn1/2^{DKO} mice vs Ctrl). (D) Serum insulin concentrations (n=7-24/group) measured during in vivo glucose-stimulated insulin release testing in 8-week-old Ctrl, β -Mfn1^{KO}, β -Mfn2^{KO} and β -Mfn1/2^{DKO} littermates. *p<0.05 by ANOVA. (E) Glucose-stimulated insulin secretion following static incubations in 2mM and 16.7 mM glucose, performed in isolated islets of 8-week-old Ctrl, β -Mfn1^{KO}, β -Mfn2^{KO} and β -Mfn1/2^{DKO} littermates. n=8-16/group. *p<0.05 by ANOVA. (F) Pancreatic β -cell mass (n=4-10/group) measured in 10-week-old Ctrl, β -Mfn1^{KO}, β -Mfn2^{KO} and β -Mfn1/2^{DKO} littermates.

Figure 2

Combined Mfn1 and Mfn2 deficiency reduces mitochondrial respiration and leads to mitochondrial fragmentation in β -cells. (A) OCR measured following exposure to 2mM glucose, 20mM glucose, and 2 μ M antimycin A in islets isolated from 8-10-week-old Ctrl and β -Mfn1/2DKO mice by a Seahorse flux analyzer. n=8-9/group. *p<0.05 by ANOVA. (B) Representative transmission EM images of β -cells from 12-week-old Ctrl, β -Mfn1KO, β -Mfn2KO and β -Mfn1/2DKO littermates. n=3/group. Mitochondria are highlighted with red arrows. (C) Quantification of mitochondrial morphology in Ctrl, β -Mfn1KO, β -Mfn2KO and β -Mfn1/2DKO β -cells from transmission EM images (~125 independent mitochondria scored/animal). n=3/group. *p<0.05 by ANOVA. (D) Imaris® generated threedimensional reconstruction of deconvolution immunofluorescence Z-stack images at 100X magnification stained for SDHA (see inset image – red) from pancreatic sections of Ctrl, β -Mfn1KO, β -Mfn2KO and β -Mfn1/2DKO mice. β -cells were

identified by insulin co-staining (inset: insulin – green). Each unique color represents a separate β -cell mitochondrial network cluster. Representative image of 6 independent mice/group. (E) β -cell mitochondrial morphology and network analysis of deconvolution immunofluorescence Z-stack images from studies depicted in Figure 2D, stained for SDHA (and insulin) from pancreatic sections of Ctrl, β -Mfn1KO, β -Mfn2KO and β -Mfn1/2DKO mice by MitoAnalyzer. n=6/group (~150 β -cells/animal were quantified). *p<0.05 by ANOVA.

Figure 3

Mfn1/2-deficient β -cells exhibit reduced mtDNA content. (A) Relative mtDNA content measured by qPCR (normalized to nuclear DNA expression) in isolated islets of 8- week-old Ctrl, β -Mfn1KO, β -Mfn2KO and β -Mfn1/2DKO littermates. n=5-7/group. *p<0.05 by ANOVA. (B) Heatmap representing relative expression (compared to littermate controls; Ctrl) of mitochondrial encoded transcripts in islets isolated from β -Mfn1KO, β -Mfn2KO and β -Mfn1/2DKO mice. n=4-6/group. §* Benjamini-Hochberg FDR (Padj) <0.05

comparing β -Mfn2KO($\S$) and β -Mfn1/2DKO($\ast$) to Ctrl. (C) Expression of OXPHOS subunit proteins by WB in islets isolated from 8-10-week-old Ctrl, β -Mfn1KO, β -Mfn2KO and β -Mfn1/2DKO mice. Representative of 4 independent mice/group. (D) OXPHOS subunit densitometry (normalized to Vinculin) from studies in Figure 3C. n=4/group; $\ast p < 0.05$ by ANOVA.

Figure 4

A single allele of Mfn2, but not Mfn1, maintains glycemic control by preserving β - cell mtDNA content despite mitochondrial fragmentation. (A) Imaris® generated three-dimensional reconstruction of deconvolution immunofluorescence Z-stack images at 100X magnification stained for SDHA (see inset image – red) from pancreatic sections of Ctrl, β - Mfn1/2DKO, β -Mfn1^{+/-}-Mfn2KO, and β -Mfn1KOMfn2^{+/-} mice. β -cells were identified by insulin co-staining (inset: insulin – green). Each unique color represents a separate β -cell mitochondrial network cluster. Representative image of 6 independent mice/group. (B) β - cell mitochondrial morphology and network analysis of deconvolution immunofluorescence Z-stack images from pancreatic sections stained for insulin and SDHA of Ctrl, β - Mfn1/2DKO, β -Mfn1^{+/-}-Mfn2KO, and β -Mfn1KOMfn2^{+/-} mice by MitoAnalyzer. n=6/group (50-200 β -cells/animal were quantified).

* $p < 0.05$ by ANOVA. (C) Blood glucose concentrations measured during IPGTT of 8-week-old Ctrl, β -Mfn1/2DKO, β -Mfn1+/-Mfn2KO, and β -Mfn1KOMfn2+/- littermates. $n = 13$ -28/group. ($p < 0.05$ by ANOVA for β -Mfn1+/- Mfn2KO and β -Mfn1/2DKO mice vs Ctrl). (D) Serum insulin concentrations ($n = 11$ -24/group) measured during in vivo glucose-stimulated insulin release testing in 8-week-old Ctrl, β -Mfn1/2DKO, β -Mfn1+/-Mfn2KO, and β -Mfn1KOMfn2+/- littermates. * $p < 0.05$ by ANOVA. (E) Relative mtDNA content measured by qPCR (normalized to nuclear DNA expression) in isolated islets of 8-week-old Ctrl, β -Mfn1/2DKO, β -Mfn1+/-Mfn2KO, and β -Mfn1KOMfn2+/- littermates. $n = 5$ -7/group. * $p < 0.05$ by ANOVA. Of note, studies in Ctrl and β -Mfn1/2DKO mice were performed together alongside all β -Mfn1KO, β -Mfn2KO, β -Mfn1+/-Mfn2KO, and β -Mfn1KOMfn2+/- littermates and thus may appear twice for purposes of relevant comparisons (in Figures 1C, 1D, 2D, 2E, 3A and again in Figures 4AE).

Figure 5

Mitofusin agonists restore mtDNA depletion following Mfn2 deficiency independent of changes in mitochondrial structure. (A) Relative mtDNA content measured by qPCR (normalized to nuclear DNA expression) from isolated islets of Ctrl and β -Mfn2KO mice following treatment with vehicle (Veh) or 0.5 μM Mfn agonists for 24 hrs. $n = 4$ /group. * $p < 0.05$ by ANOVA. (B) Expression of Mfn1 and Mfn2 by WB in islets isolated from 10-12-week-old db/+ and db/db mice. Vinculin and cyclophilin B serve as loading

controls. Representative image (left) of 3-4 independent mice/group. Mfn1 and 2 densitometry, normalized to vinculin or cyclophilin B (right). n= 3-4/group; *p<0.05 by two-tailed t-test. (C) Relative mtDNA content measured by qPCR (normalized to nuclear DNA expression) from isolated islets of 10-12-week-old db/+ and db/db mice following treatment with vehicle (Veh) or 0.5 μ M mitofusin agonists for 24 hrs. n=5-6/group. *p<0.05 by two-tailed t-test. (D) β -cell mitochondrial morphology and network analysis by MitoAnalyzer of deconvolution immunofluorescence Z-stack images, stained for SDHA (and insulin), from pancreatic islets of 10- 12-week-old db/+ and db/db mice following treatment with vehicle (Veh) or 0.5 μ M mitofusin agonists for 24 hrs. n=4-6/group (~150- 200 β -cells/animal were quantified). (E) Insulin secretion index from isolated islets of 10-12-week-old db/+ and db/db mice following treatment with vehicle (Veh) or 0.5 μ M mitofusin agonists for 24 hrs. n=5-6/group. *p<0.05 by two-tailed t-test.

Figure 6

Mfn1/2-deficiency induces expression of genes associated with mtDNA replication. (A) Hierarchical clustering of RNAseq samples displaying the 500 genes with the highest mean expression in islets of 12-week-old mice. n=4-6/group. (B) Pathway analysis of top differentially regulated pathways in β -Mfn1/2DKO islets compared to littermate Ctrl islets, demarcated by both false discovery rate (FDR) and the number of significantly differentially expressed genes (DEGs) per pathway. n=4-6/group. (C) Volcano plot depicting differential RNA expression in islets of β -Mfn1/2DKO mice compared to littermate Ctrl

mice. Significantly differentially expressed genes demarcated by $-\log_{10}$ FDR $>$ or $<$ 2 and $\log_2$ fold change (FC) $>$ or $<$ 0.6. $n=4-6$ /group. (D) Volcano plot depicting differential RNA expression of MitoCarta 2.0 targets in islets of β -Mfn1/2DKO mice compared to littermate Ctrl mice. Significantly differentially expressed genes demarcated by $-\log_{10}$ FDR $>$ or $<$ 2 and $\log_2$ fold change (FC) $>$ or $<$ 0.6. $n=4-6$ /group. (E) Differential RNA expression heatmap of selected genes from islets of β -Mfn1KO, β -Mfn2KO and β -Mfn1/2DKO mice compared to littermate Ctrl mice. up. $\ddagger$ §* Benjamini-Hochberg FDR (Padj) $<$ 0.05 comparing β -Mfn1KO($\ddagger$), β -Mfn2KO($\S$) and β -Mfn1/2DKO($*$) to Ctrl. $n=4-6$ /group.

Figure 7

Mfn1 and 2 maintain expression of Tfam in β -cells. (A) Expression of Tfam, key replisome proteins, and LonP1 by Western blot (WB) in islets isolated from 11- week-old Ctrl, β -Mfn1KO, β -Mfn2KO and β -

Mfn1/2DKO mice. Representative of 4 independent mice per group. (B) Densitometry (normalized to Vinculin) from studies in Figure 7A. $n=4/\text{group}$; $*p<0.05$ by ANOVA. (C) Quantification of single strand mtDNA products measured by qPCR in isolated islets from 8-weekold Ctrl, β -Mfn1KO, β -Mfn2KO and β -Mfn1/2DKO littermates. $n=5-13/\text{group}$. $*p<0.05$ by ANOVA. (D) Deconvolution immunofluorescence image at 100X magnification of islets from Ctrl and β -Mfn1/2DKO mice stained for Ssbp1 (red), mtDNA (green), and Pdx1 (blue). Representative of 8 independent experiments. White arrows demarcate co-localized Ssbp1+ mtDNA+ structures. (E) Quantification of mitochondrial nucleoid Ssbp1 localization (Ssbp1+ mtDNA+ co-localization) in Ctrl and β -Mfn1/2DKO β -cells from studies depicted in Figure 7D by Pearson's correlation coefficient (PCC). $n=8/\text{group}$. $*p<0.05$ by two-tailed t-test. (~ 100 β -cells from each animal per group were analyzed). (F) Quantification of nuclear Ssbp1 localization in Ctrl and β -Mfn1/2DKO β -cells from studies depicted in Figure 7D by Pearson's correlation coefficient (PCC). $n=8/\text{group}$. $*p<0.05$ by two-tailed t-test. (~ 100 β -cells from each animal per group were analyzed). (G) Imaris® generated 3D reconstruction of deconvolution immunofluorescence Z-stack images at 100X magnification stained for DNA of β -cells (see inset image – Pdx1, blue, DNA, green) from Ctrl and β -Mfn1/2DKO mice. Colors represent relative mitochondrial volume. Representative image of 8 independent mice/group. (H) Quantification of relative nucleoid volume of Ctrl and β -Mfn1/2DKO β -cells from studies depicted in Figure 7G. $n=8/\text{group}$. $*p<0.05$ by two-tailed t-test. (~ 100 β -cells from each animal per group were analyzed).

Figure 8

Mfn1 and 2 maintain β -cell function through Tfam-mediated mtDNA copy number control. (A) Expression of mouse-specific Tfam and human-specific TFAM by WB in islets isolated from 10-15-week-old Ctrl and β -Mfn1/2DKO mice, transduced with empty vector control (Ad.EV) or human TFAM-overexpressing (Ad.hTFAM) adenoviral particles. Representative of 4 independent mice/group. (B) Densitometry (normalized to cyclophilin B) from studies in Figure 8A. $n=4$ /group; $p=NS$ (non-significant) by t-test. (C) Glucose-stimulated insulin secretion following static incubations in 2mM and 16.7 mM glucose, performed in isolated Ctrl and β -Mfn1/2DKO islets following transduction with Ad.EV or Ad.hTFAM adenoviral particles. $n=4$ /group. $*p<0.05$ by ANOVA. (D) Relative mtDNA content measured by qPCR (normalized to nuclear DNA expression) from isolated Ctrl and β -Mfn1/2DKO islets following transduction with Ad.EV or Ad.hTFAM adenoviral particles. $n=8-10$ /group. $*p<0.05$ by ANOVA.

Supplementary Files

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- [SidaralaSupplementarytable1.pdf](#)
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