Cisd2 modulates the differentiation and functioning of adipocytes by regulating intracellular \( \text{Ca}^{2+} \) homeostasis

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**CISD2** is a causative gene associated with Wolfram syndrome (WFS). However, it remains a mystery as to how the loss of CISD2 causes metabolic defects in patients with WFS. Investigation on the role played by Cisd2 in specific cell types may help us to resolve these underlying mechanisms. White adipose tissue (WAT) is central to the maintenance of energy metabolism and glucose homeostasis in humans. In this study, adipocyte-specific Cisd2 knockout (KO) mice showed impairment in the development of epididymal WAT (eWAT) in the cell autonomous manner. A lack of Cisd2 caused defects in the biogenesis and function of mitochondria during differentiation of adipocytes *in vitro*. Insulin-stimulated glucose uptake and secretion of adiponectin by the Cisd2 KO adipocytes were decreased. Moreover, Cisd2 deficiency increased the cytosolic level of \( \text{Ca}^{2+} \) and induced \( \text{Ca}^{2+} \)-calcineurin-dependent signaling that inhibited adipogenesis. Importantly, Cisd2 was found to interact with Gimap5 on the mitochondrial and ER membranes and thereby modulate mitochondrial \( \text{Ca}^{2+} \) homeostasis in adipocytes. Thus, it would seem that Cisd2 plays an important role in intracellular \( \text{Ca}^{2+} \) homeostasis, which is required for the differentiation and functioning of adipocytes as well as the regulation of glucose homeostasis in mice.

**INTRODUCTION**

CISD2, CDGSH iron–sulfur cluster-containing protein 2, is the second causative gene associated with Wolfram syndrome (WFS), which is an autosomal-recessive neurodegenerative disorder with highly variable clinical manifestations, including diabetes insipidus, diabetes mellitus, optic atrophy and deafness \((1)\). Cisd2 is located at the ER \((1,2)\) or mitochondrial membranes \((3)\) and forms a homodimer harboring two redox-active 2Fe–2S clusters \((4)\). Previously, we established a conventional Cisd2 knockout (KO) and a transgenic mouse models that demonstrated the role of Cisd2 in maintaining the integrity and functioning of mitochondria, the regulation of mammalian life span and the maintenance of a healthy life \((3,5)\). In addition, Cisd2 may be involved in the Bcl-2-mediated regulation of autophagy and \( \text{Ca}^{2+} \) homeostasis that maintains the structure and performance of skeletal muscle \((2,6)\). A recent study suggested that Cisd2 might act as a redox protein related to the regulation of the unfolded protein response and redox status in human cells \((7)\). However, the biological functions of Cisd2 *per se* are not well defined. Because of its importance in maintaining ER and mitochondrial function, Cisd2 deficiency is likely to exert different...
effects on different cell types. When considering the role of WFS in the systemic metabolic regulation, it is important to explore the physiological functions of Cisd2 and the mechanisms by which Cisd2 regulates glucose homeostasis in specific cell types.

White adipose tissue (WAT) is able to secrete a number of cytokines, known as adipokines, which are involved in the maintenance of glucose homeostasis and the regulation of energy metabolism (8–10). Defects in the formation and function of WAT are known to result in abnormal glucose homeostasis, insulin insensitivity and/or type 2 diabetes in mice (11–14). Thus, exploration of the pathways that control adipocyte differentiation and/or the secretion of adipokines should provide useful information that will help the development of new strategies for the prevention and treatment of metabolic disorders.

Recent studies have defined the zone between mitochondria and the ER, which is called mitochondria-associated ER membranes (MAM), as an area crucial for the communication between the two organelles. Such communication includes the highly efficient transmission of physiological and pathological signals between the ER and the mitochondria that are mediated by Ca$^{2+}$ (15). Coupling at the ER-mitochondria interface is important for the regulation of intracellular Ca$^{2+}$ homeostasis during cellular metabolism, cell survival and the pathogenesis of various diseases such as Alzheimer’s disease and type 2 diabetes (16–18). In addition, mitochondria are able to modulate both the amplitude and the spatio-temporal pattern of intracellular Ca$^{2+}$ changes (19). The Ca$^{2+}$ buffering capacity of mitochondria plays a crucial role in the regulation of the Ca$^{2+}$-dependent signaling and pathogenesis of a wide range of diseases (20,21).

In the present study, we demonstrated that Cisd2 deficiency leads to defects in the differentiation and function of adipocytes both in vivo and in vitro. In addition, we showed for the first time that Cisd2 and its interacting protein, Gimap5, might additively regulate the Ca$^{2+}$ buffering activity of mitochondria that maintains intracellular Ca$^{2+}$ homeostasis. Loss of Cisd2 increased calcineurin activity and the level of cytosolic Ca$^{2+}$, which then interrupted the signaling cascade that is involved in the differentiation of adipocytes.

RESULTS

Cisd2 deficiency impairs the development of eWAT in mice

To understand the mechanism underlying systemic glucose intolerance in WFS, we focused on investigating abnormalities affecting adipose tissues using a mouse model. We observed that total body fat was significantly decreased in 12-week-old Cisd2 KO mice compared with their age-matched wild-type (WT) control mice (Fig. 1A). To explore whether this defect acted in a cell autonomous manner, we investigated the phenotypes of adipose tissues in adipocyte-specific Cisd2 KO mice (aKO) that were generated by crossing the mice carrying the Cisd2 floxed allele (Cisd2$^{f/f}$) with aP2-Cre transgenic mice (Supplementary Material, Fig. S1A–E). It was found that the Cisd2 aKO mice also showed a decrease in the total body fat at 12 weeks and the level of body fat loss was equivalent to that of the Cisd2 KO mice (Fig. 1A). These results suggest that Cisd2 may play a cell autonomous role in the development of adipose tissues in mice.

Next, we assessed the phenotype of mice at a younger age and examined their epidymal WAT (eWAT), one of the various WATs found in the visceral region; we targeted this area because a defect in visceral fat is thought to be more closely related to metabolic disorders (22). First, it was found that the expression of Cisd2 in the eWAT of the WT mice was induced at 2 weeks and that this increase became more pronounced during the maturation of eWAT in mice (Fig. 1B–D), which suggests that Cisd2 is important for the development of eWAT. Furthermore, the size and weight of eWAT were both significantly lower in Cisd2 KO mice at 8 weeks (Fig. 1E). Histological examination showed that the size of eWAT adipocytes and the amount of intracellular lipids present in the eWAT adipocytes increased in parallel with the age of the WT mice. However, when compared with the control WT mice at 8 weeks, it was found that the Cisd2 KO mice had smaller adipocytes and that there were fewer lipid droplets present in the eWAT, as revealed by hematoxylin–eosin (H&E) staining and immunofluorescent staining (Fig. 1F). The expression levels of various adipogenic genes (C/EBPα, PPARγ, aP2 and adiponectin) were found to be down-regulated significantly in the eWAT of the Cisd2 KO mice at 8 weeks (Fig. 1G) compared with wild-type control mice. Furthermore, the findings when H&E-stained sections of aKO mice and Cisd2$^{f/f}$ control mice at 4 weeks were compared, it revealed that there was no significant difference in the size of the eWAT adipocytes (Fig. 1H). However, at 12 weeks, the eWAT adipocytes of aKO mice were smaller than those of Cisd2$^{f/f}$ mice (Fig. 1H).

In parallel, the expression levels of various adipogenic genes (Fig. 1I; Supplementary Material, Fig. S1F and G) in eWAT of the aKO mice were found to be significantly lower than those of the control Cisd2$^{f/f}$ mice. This decline was found to correlate well with the efficiency at which the Cisd2 gene in eWAT had been deleted at 12 weeks (Supplementary Material, Fig. S1F and G).

The commitment to differentiate into eWAT and the maturation of eWAT was then examined to determine whether Cisd2 is involved in these processes. The mRNA expression levels of preadipocyte factor 1 (Pref-1) and aP2 in eWAT were measured in mice at different ages. It was found that the mRNA expression level of aP2 in the eWAT of Cisd2 KO mice at 4 weeks was lower than that of the WT mice, but there was no significant difference in the mRNA expression level of Pref-1 (Fig. 1J). Furthermore, no increase in the level of aP2 expression was found to occur as the Cisd2 KO mice aged (Fig. 1J). Taken together, these findings support the hypothesis that a lack of Cisd2 leads to a cell autonomous defect in the maturation of eWAT in mice.

Cisd2 is essential for differentiation of adipocytes in vitro

To further delineate the cell autonomous role of Cisd2 in white adipogenesis, we used a well-established differentiation platform that produces white adipocytes from pluripotent mouse embryonic fibroblasts (MEFs) and 3T3-L1 preadipocytes (23,24), respectively. The results showed that the mRNA and protein expression levels of Cisd2 were increased during the early stage of adipogenic induction and are maintained at high levels throughout the remaining differentiation process of the MEFS (Fig. 2A and B) and 3T3-L1 preadipocytes (Supplementary Material, Fig. S2A and B). These findings indicate that Cisd2 is likely to
Figure 1. Cisd2 deficiency impairs white adipogenesis in mice. (A) Micro-CT analysis showing the total body fat in WT, Cisd2 KO, and adipocyte-specific Cisd2 KO (aKO) male mice at 12-weeks. V, ventral; D, dorsal; L, left; R, right. (B–D) The mRNA (B) and protein (C, D) expression levels of Cisd2 in the eWAT of WT male mice at the indicated ages. (E) The size and weight of eWAT in WT and Cisd2 KO male mice at 8 weeks. (F) Sections of eWAT from male mice at 2 weeks and 8 weeks were subjected to H&E staining and to immunofluorescence staining using an antibody specific to perilipin, a lipid droplet-associated protein. Scale bar = 50 μm. (G) Using Taqman probe-based Q-PCR analysis, the mRNA levels of the indicated genes were determined in eWAT from WT and Cisd2 KO male mice at 8 weeks. The expression level was normalized against β actin. (H) H&E staining of sections of eWAT in Cisd2f/f (f/f) and aKO male mice at 4 and 12 weeks. Scale bar = 50 μm. (I) The mRNA expression levels of the indicated genes in eWAT of the Cisd2f/f and aKO male mice at 12 weeks. (J) The mRNA expression levels of aP2 and preadipocyte factor-1 (Pref-1) in the eWAT of Cisd2 WT and KO male mice at the indicated ages. All the data are presented as means ± SD (*, P < 0.05).
Figure 2. Cisd2 deficiency impairs white adipogenesis in MEFs. (A) The mRNA expression levels of Cisd2 at Days 0, 0.5, 1, 3 and 9 after induction of white adipogenesis in WT MEFs. (B) The protein expression levels of Cisd2, Pref-1 and aP2 at Days 0, 0.5, 1, 3 and 9 after induction of white adipogenesis in WT MEFs. (C and D) After 9 days of adipogenic induction, the adipocytes from WT MEFs (WT-D9d) and Cisd2 KO MEFs (KO-D9d) were stained with Oil Red O (C) and the amount of intracellular lipids was determined (D). Scale bar = 50 μm. (E) The mRNA expression levels of the indicated genes in adipocytes differentiated from WT and Cisd2 KO MEFs. (F and G) The mRNA levels of aP2 (F) and Pref-1 (G) at Days 0, 1, 3 and 9 after adipogenic induction in WT and Cisd2 KO MEFs. (H) The basal and insulin-stimulated glucose uptake was measured in differentiated adipocytes treated without and with insulin, respectively. (I) Adiponectin secreted from adipocytes was measured by an ELISA kit. All the data are presented as means ± SD (**P < 0.05; ***P < 0.005).
play a key role in the promotion of the differentiation and maturation of adipocytes.

To examine whether Cisd2 is required for adipogenesis in vitro, adipogenic induction of precursor cells with or without the presence of Cisd2 was explored. To do this, we isolated fresh MEFs from Cisd2 KO and WT mouse embryos. The results revealed that, at 9 days after adipogenesis, there was a lower intracellular lipid content (Fig. 2C and D) and decreased expression levels of various adipogenic genes (Fig. 2E) in the adipocytes differentiated from Cisd2 KO MEFs compared with control WT MEFs. In addition, the mRNA level of ap2 was found to be decreased in Cisd2 KO MEFs at Days 3 and 9 after adipogenic induction compared with the control MEFs (Fig. 2F). However, there was no significant difference in the level of Pref-1 between the WT and Cisd2 KO MEFs during adipogenesis (Fig. 2G). These findings indicate that it is the maturation of adipocytes that is defective. In addition, we examined whether this impairment influences the physiological functioning of these adipocytes. The results showed that there was a decrease in insulin-stimulated glucose uptake (Fig. 2H) and a decreased secretion of adiponectin by the Cisd2 KO adipocytes compared with WT controls (Fig. 2I). In addition, we found similar results during the differentiation of 3T3-L1 cells that had undergone Cisd2 knockdown (Supplementary Material, Fig. S2C–I). These results together indicate that Cisd2 deficiency affects the differentiation/maturation and functioning of adipocytes in vitro.

Cisd2 deficiency leads to defects in the biogenesis and functioning of mitochondria during adipogenesis

It has been reported that an increase in mitochondrial biogenesis and functioning is a hallmark of white adipogenesis and that these increases are required for the physiological function of mature adipocytes during energy metabolism and the regulation of glucose homeostasis (25,26). Based on these findings, we examined the effect of Cisd2 deficiency on the induction of the biogenesis and functioning of mitochondria during white adipogenesis. The results showed that the mRNA expression levels of various genes involved in the regulation of mitochondrial biogenesis, such as PGC-1α and Tfam, were highly increased in WT MEFs after induction of adipogenesis (Fig. 3A and B, white bar). However, this induction was impaired in MEFs where there was a deficiency in Cisd2 (Fig. 3A and B, black bar). In addition, after adipogenesis, the elevation in the level of some subunits of respiratory enzymes and of citrate synthase activity were both compromised in Cisd2 KO MEFs compared with WT MEFs (Supplementary Material, Fig. S3A and B). These defects in mitochondrial biogenesis directly led to deficiencies in the activities of various respiratory enzyme complexes, including Complex I–III and Complex IV (Supplementary Material, Fig. S3C and D).

Next, we measured the bioenergetic functioning of mitochondria using a Seahorse XF-24 analyzer. The results showed that ATP-coupled respiration and the reserve capacity (RC) of the mitochondria were slightly decreased in undifferentiated Cisd2 KO MEFs compared with undifferentiated WT MEFs (Fig. 3C and D). After 9 days of adipogenesis, the two functional parameters were even more dramatically decreased in adipocytes derived from Cisd2 KO MEFs compared with WT MEFs (Fig. 3E and F). Assays of the glutamate/malate-supported and succinate-supported State 3 respiration rates (G/M + ADP and Su + ADP) were also carried out and revealed a decrease in undifferentiated Cisd2 KO MEFs compared with undifferentiated WT MEFs, and, again, this decrease was more pronounced after induction of adipogenesis (Supplementary Material, Fig. S3E and F). Morphologically, a detailed transmission electron microscopy (TEM) examination was carried out and this further revealed the presence of abnormal mitochondria that had marked swelling; this was associated with disarrayed or disappeared cristae in the eWAT of Cisd2 KO mice at 8 weeks compared with eWAT from WT mice (Fig. 3G). All of these findings suggest that a deficiency in Cisd2 impairs the induction of mitochondrial biogenesis during adipogenesis and, furthermore, leads to defects in ultrastructure and bioenergetics functioning of mitochondria. These changes are likely to contribute to the physiological defects that are observed in the mature adipocytes derived from Cisd2 KO MEFs.

Cisd2 deficiency increases cytosolic Ca2+ and impairs the Ca2+ buffering capability of mitochondria

It has been reported that the loss of Cisd2 results in impairment of intracellular Ca2+ homeostasis in primary myoblasts and in differentiated myotubes (6). By monitoring the Ca2+ levels of single cells using Fura-2/AM staining, we observed that there was a higher level of Ca2+ in the cytosol of MEFs lacking Cisd2 (Fig. 4A). In addition, we assessed the ER-stored and mitochondria-stored Ca2+ ions using ER-targeted and mitochondria-targeted aequorin (erAEQ and mtAEQ). After binding with a prosthetic group, coelenterazine, the active aequorin is able to react with Ca2+ and oxidize the prosthetic group, which results in the emission of light (27). The location of ectopically expressed erAEQ and of ectopically expressed mtAEQ in MEFs was confirmed to be as expected (Supplementary Material, Fig. S4A and B). The results showed that the Ca2+ level in ER was similar when MEFs with or without Cisd2 were compared; however, the mitochondrial Ca2+ level was significantly decreased in Cisd2 KO MEFs compared with WT MEFs (Fig. 4B). Supporting this, we also found that the Ca2+ content of mitochondria was lower in Cisd2 KO MEFs compared with WT MEFs when the mitochondrial calcium indicator Rhod-2/AM was used with single cells (Fig. 4C). However, in contrast, there was no obvious difference in the cardiolipin content of mitochondria between WT and Cisd2 KO MEFs (Supplementary Material, Fig. S4C), which indicates that the effect of Cisd2 KO on MEF mitochondria is not due to a change in mitochondrial mass.

To examine the involvement of Cisd2 in the regulation of mitochondrial Ca2+ uptake, we measured the change in mitochondrial Ca2+ after thapsigargin (TG) treatment; this was done by staining with Rhod-2/AM based on the fact that there is consistency between the results obtained previously using Rhod-2/AM staining and using the aequorin assay. Our findings showed that the level of mitochondrial Ca2+ was increased by up to 1.6-fold in the WT MEFs after TG stimulation. On the other hand, there was only a 1.35-fold increase in mitochondrial Ca2+ after TG induction when Cisd2 KO MEFs were used (Fig. 4D and E). To validate the role of Cisd2 in promotion of mitochondrial Ca2+ uptake, we monitored changes in mitochondrial Ca2+ in WT and Cisd2 KO MEFs that were overexpressing green fluorescent
Figure 3. Cisd2 deficiency impairs induction of mitochondrial biogenesis and function during white adipogenesis of MEFs. (A and B) The mRNA levels of PGC-1α (A) and Tfam (B) in WT and Cisd2 KO MEFs before and after 9 days of adipogenic differentiation (D9d). (C and D) OCRs of undifferentiated WT and Cisd2 KO MEFs were monitored (C) and quantified (D) using a Seahorse XF24 analyzer. The indicated chemicals (OA, oligomycin A; DNP, 2,4-dinitrophenol; AA, antimycin A), were added sequentially to determine the ATP-coupled respiration rate, reserve capacity (RC), and the non-mitochondrial respiration rate, respectively. (E and F) OCRs of adipocytes differentiated from WT and Cisd2 KO MEFs (WT-D9d and KO-D9d) were monitored (E) and quantified (F) using a Seahorse XF24 analyzer. (G) TEM examination for the ultrastructure of the eWAT in the WT and Cisd2 KO mice at 8 weeks. M, mitochondria; V, vacuole; DM, degenerating mitochondria; CD, cristae degeneration and MF, myelin figure, which is the membranous debris of mitochondrial degeneration. All the data are presented as means ± SD (*, P < 0.05).
Figure 4. Cisd2 regulates Ca\(^{2+}\) homeostasis by promoting mitochondrial Ca\(^{2+}\) uptake. (A) The level of cytosolic Ca\(^{2+}\) in single cells (n = 50) was measured by fluorescence microscopy using Fura-2/AM staining. (B) WT and Cisd2 KO MEFs were transfected with mitochondria-targeted (mtAEQ) or ER-targeted (erAEQ) aequorin. After the active form of aequorin had reacted with any Ca\(^{2+}\) present in the organelles, the emitted luminescence was determined by a luminator. (C) The level of mitochondrial Ca\(^{2+}\) in single cells (n = 45) was measured by fluorescence microscopy using Rhod-2/AM staining. (D) The levels of mitochondrial Ca\(^{2+}\) in single cells before and after TG treatment were monitored over time by fluorescence microscopy using Rhod-2/AM staining. (E) Representative images obtained by fluorescence microscopy are presented that show the intensity of Rhod-2 before (0 s) and after TG treatment (150 s) in WT and Cisd2 KO MEFs. Scale bar = 100 \(\mu\)m. The increase in the level of mitochondrial Ca\(^{2+}\) after 150 s of TG treatment was measured. (F) The levels of mitochondrial Ca\(^{2+}\) before and after TG treatment were monitored in WT and Cisd2 KO MEFs transfected with the scramble vector (Scr) or GFP-Cisd2. (G) The increase in the level of mitochondrial Ca\(^{2+}\) after 150 s of TG treatment was determined. All the data are presented as means ± SD (*, P < 0.05).
protein (GFP)-tagged Cisd2 (GFP-Cisd2) or His-tagged Cisd2 (His-Cisd2) (Supplementary Material, Fig. S4D and E). The findings indicated that the decline in mitochondrial Ca\(^{2+}\) uptake in Cisd2 KO MEFs was recovered to the comparable level of WT MEFs after there had been reexpression of Cisd2 (Fig. 4F and G; Supplementary Material, Fig. S4F and G). These findings imply that Cisd2 plays an important role in the regulation of Ca\(^{2+}\) homeostasis in MEFs and does this by being involved in increasing the uptake and accumulation of Ca\(^{2+}\) by the mitochondrion.

Inhibition of Ca\(^{2+}\)-calcineurin signaling restores adipogenesis in Cisd2-deficient MEFs

A number of studies have demonstrated that Ca\(^{2+}\)-dependent signaling elicited by an increase in cytosolic Ca\(^{2+}\) level is able to potently inhibit adipogenesis (28,29). Here, we investigated the contribution of the higher cytosolic Ca\(^{2+}\) levels caused by Cisd2 deficiency make to the inhibition of adipogenesis. We observed that the decrease in the lipid content during adipogenesis of adipocytes that had been differentiated from Cisd2 KO MEFs was recovered after treatment with 0.5 \(\mu\text{M}\) BAPTA, a Ca\(^{2+}\)-chelator. This recovery was even more conspicuous after the addition of 1 \(\mu\text{M}\) BAPTA (Fig. 5A). It was also found that the activity of calcineurin, a Ca\(^{2+}\)-dependent phosphatase, was significantly elevated in the eWAT of Cisd2 KO mice at 12 weeks (Fig. 5B) and in Cisd2 KO MEFs (Fig. 5C) as compared with their equivalent WT controls.

To examine the involvement of calcineurin, MEFs were treated with two calcineurin inhibitors, FK-506 and calcineurin-inhibitory peptide (CaNi), during adipogenesis. The ability of the MEFs to undergo adipocyte differentiation was then evaluated. Cisd2 KO MEFs that had been treated with 2.5 ng/ml FK or 1 \(\mu\text{M}\) CaNi during adipogenic induction showed restoration of the number of adipocytes produced by Cisd2 KO MEFs, of the lower intracellular lipid content of adipocytes produced from Cisd2 KO MEFs (Fig. 5D) and of the lower expression levels of various adipogenic genes by the adipocytes produced from Cisd2 KO MEFs (Fig. 5E and F). Furthermore, the defects found in the induction of mitochondrial biogenesis and in respiratory functioning were also significantly reversed when calcineurin was inhibited in adipocytes differentiated from Cisd2 KO MEFs (Fig. 5F–H). Furthermore and in agreement with the above results, the impairment of adipocyte differentiation in 3T3-L1 cells with Cisd2 knockdown was also restored when calcineurin was inhibited by either FK-506 or cyclosporine A (CsA) (Supplementary Material, Fig. S5).

These findings together indicate that the increase in the cytosolic Ca\(^{2+}\) and calcineurin activity found in Cisd2 KO MEFs is likely to be involved in the interruption of the signaling cascade that is activated during adipogenesis.

Cisd2 interacts with Gimap5 in the MAM and additively regulates mitochondrial Ca\(^{2+}\) uptake

To gain further insights into the molecular mechanism by which Cisd2 regulates mitochondrial Ca\(^{2+}\) uptake, a search was carried out to identify Cisd2-interacting proteins. HEK-293T cells were transfected with a GFP-Cisd2 construct. Total proteins were immunoprecipitated with an anti-GFP antibody, and the precipitate was analyzed for Cisd2-interacting proteins by silver staining and LC-MS/MS (Fig. 6A and Supplementary Material, Table S1). One protein, GTPase of immune-associated protein 5 (Gimap5), was identified as a promising Cisd2-interacting candidate among all identified proteins; this was based on known information about its subcellular location (30–32). The LC-MS/MS results were confirmed by observing that Gimap5 is able to be immunoblotted after GFP-Cisd2 had been immunoprecipitated from the total protein lysate of HEK-293T cells (Fig. 6B). When immunofluorescent images of transfected MEF cells were analyzed, it was found that endogenous Gimap5 (red) was partially colocalized with GFP-Cisd2 (green) (Fig. 6C). Furthermore, an endogenous coimmunoprecipitation assay revealed that Cisd2 was able to be detected simultaneously after a pull-down of Gimap5 in MEFs (Fig. 6D). To examine directly the interaction between these two proteins in cells, we performed a split YFP assay. Cisd2 and Gimap5 were fused with the C-terminal (VC) or N-terminal (VN) domain of Venus, an YFP, respectively, and these constructs were then expressed in HEK-293T cells in order to evaluate by monitoring of the YFP signal whether there is interaction between Cisd2 and Gimap5. Using a confocal microscope, the fluorescent images revealed that YFP signal was present in the cytosol and not in the nucleus. This signal was mainly colocalized with mitochondria when cells were concurrently expressing Cisd2-VC and VN-Gimap5 (Fig. 6E and Supplementary Material, Fig. S6A). Moreover, subcellular fractionation showed that both Cisd2 and Gimap5 were enriched in the MAM region of MEFs (Fig. 6F). These findings suggest that Cisd2 and Gimap5 interact with each other and that this occurs in the MAM, in particular, of cells.

Interestingly, when the expression of Gimap5 in Cisd2-deficient MEFs and of Cisd2 in Gimap5-deficient MEFs was explored, it was revealed that there was a compensatory up-regulation of the other protein in the MEFs (Supplementary Material, Fig. S6B–E). Furthermore, we observed that a loss of Gimap5 also resulted in a decrease in the Ca\(^{2+}\) uptake of mitochondria in MEFs (Fig. 6G and H). Importantly, MEFs lacking both Cisd2 and Gimap5 showed an additive decline in Ca\(^{2+}\) uptake by mitochondria (Fig. 6G and H), which indicates that each of them is essential to the modulation of the Ca\(^{2+}\) buffering capability of mitochondria.

Lastly, we investigated whether Gimap5 also plays a role in the differentiation of adipocytes. It was found that both the mRNA and protein level of Gimap5 were upregulated within 1 day of adipogenic differentiation, which is a similar result to that is obtained for Cisd2 (Supplementary Material, Fig. S6F–H). In addition, we were also able to show that the ability to undergo adipogenic induction of MEFs with Gimap5 knockdown was impaired (Supplementary Material, Fig. S6I–L). All of these findings have substantiated the idea that Cisd2 interacts with Gimap5 and that together they contribute to the maintenance of intracellular Ca\(^{2+}\) homeostasis, which is well known to play an important role in the process of adipogenic differentiation.

**DISCUSSION**

In this study, we first established adipocyte-specific Cisd2 KO mice and demonstrated that Cisd2 is required for the differentiation and functioning of white adipocytes; furthermore, we also showed that this occurs in a cell autonomous manner. The
Figure 5. Inhibition of the Ca$^{2+}$-calcineurin pathway in MEFs with Cisd2 deficiency results in the recovery of white adipogenesis. (A) MEFs were treated with indicated concentrations of BAPTA during white adipogenesis. The intracellular lipids of the adipocytes were stained with Oil Red O and quantified. Scale bar = 50 μm. (B and C) The serine/threonine phosphatase activities of calcineurin were measured in eWAT from WT and Cisd2 aKO male mice at 12-wk (B) and in WT and Cisd2 KO MEFs (C). (D) MEFs were treated with FK-506 (2.5 ng/ml) or a cell-permeable calcineurin-inhibitory peptide (CaNi, 1 μM) during white adipogenesis. The intracellular lipids present in the adipocytes were stained with Oil Red O and quantified. Scale bar = 50 μm. (E–H) WT and Cisd2 KO MEFs were treated with or without FK-506 or CaNi during adipogenesis. After 9 days of adipogenic differentiation, the mRNA and protein levels of the various indicated genes in the cells (E–G) and glutamate and malate-supported State 3 respiration rates of the cells (H) were determined. All the data are presented as means ± SD (*, P < 0.05).
**Figure 6.** Cisd2 interacts with Gimap5 and additively modulates mitochondrial Ca\(^{2+}\) uptake. (A) Image of a silver stained SDS polyacrylamide gel showing the proteins coimmunoprecipitated with GFP-Cisd2 or GFP in HEK-293T cells. The asterisk (*) indicates the location of Gimap5, which was identified by LC-MS/MS analysis. HC, heavy chain of IgG; LC, light chain of IgG. (B) GFP-Cisd2 was expressed in HEK-293T cells and was immunoprecipitated using an anti-GFP antibody and IgG. The indicated proteins were detected by western blot analysis. (C) The location of GFP-Cisd2 and the endogenous Gimp5 in MEFs were determined by immunofluorescence staining. Using confocal microscopy, the fluorescent images show the location of GFP-Cisd2 (green) and Gimap5 (red). Scale bar = 10 μm. (D) Endogenous Gimap5 in
process of adipogenic differentiation is influenced by a variety of extrinsic factors and intracellular signaling pathways (24). Of particular interest is the effects of intracellular Ca\(^{2+}\) on adipocyte differentiation (28,29,33) and on the regulation of glucose homeostasis in humans and using animal models (34,35). In this study, we demonstrated that an elevation of cytosolic Ca\(^{2+}\) and calcineurin-dependent signaling might be the mechanism underlying the impairment of adipocyte differentiation and glucose intolerance in the mouse with Cisd2 deficiency.

Although it has been demonstrated that a loss of Cisd2 leads to dysregulation of intracellular Ca\(^{2+}\) in primary lymphoblastoid cells (1), primary myoblasts (6) and immortalized MEFs (7), a discrepancy still exists in the distribution of Ca\(^{2+}\) among various different cellular compartments. Consistent with the above findings obtained using the abovementioned primary cell cultures, we observed the presence of a higher cytosolic Ca\(^{2+}\) level in primary cultures of Cisd2 KO MEFs. Furthermore, although we also found a slight increase in ER-stored Ca\(^{2+}\), this change was not statistically significant.

Proteins localized between the ER membrane and the outer membrane of mitochondria seem to contribute to the structural stabilization of MAMs and to Ca\(^{2+}\) handling by MAMs (36). In the present study, the localization of Cisd2 suggests that Cisd2 may be involved in the functioning of MAMs, especially in signal transmission involving Ca\(^{2+}\). Importantly, we were able to extend the possible role of Cisd2 to involvement in modulating the ability of mitochondria to sequester excess cytosolic or ER-released Ca\(^{2+}\). In addition to its involvement in Ca\(^{2+}\) storage by the ER, this also provides a significant insight into the function of Cisd2 and the mechanism by which Cisd2 is involved in the handling of intracellular Ca\(^{2+}\) homeostasis. Although opposite results have been observed in immortalized MEFs compared with the abovementioned primary cell cultures (7), it is worth noting that unexpected genetic changes or genetic selection may occur during immortalization and it is possible that such changes might alter the Ca\(^{2+}\) status of cells. In addition to measuring the Ca\(^{2+}\) level in different cellular compartments, we also directly determined Ca\(^{2+}\) using a specific Ca\(^{2+}\) reporter protein rather than monitoring the Ca\(^{2+}\) released from compartments by chemical treatment. In this regard, we believe that our findings are more meaningful and reflect better the physiological role of Cisd2 in the regulation of Ca\(^{2+}\) homeostasis.

Previous studies have shown that Gimap5 is located in Golgi apparatus, in the ER, in mitochondria and in lysosomes and that it is involved in the regulation of Ca\(^{2+}\) homeostasis, in maintaining mitochondrial integrity, in ER stress and in cell survival (31,32,37–40). In this study, we demonstrated that Gimap5 seems to be located mainly in the MAM region and that it interacts with Cisd2 in MEFs. Gimap5 deficiency led to a decrease in the uptake of mitochondrial Ca\(^{2+}\), but there was no apparent apoptosis or cell death in these MEFs. Interestingly, there was an additive detrimental effect on Ca\(^{2+}\) uptake by mitochondria when Cisd2 and Gimap5 were both deficient. This implies that these two proteins form a complex and regulate Ca\(^{2+}\) homeostasis in parallel. Moreover, the upregulation of the other protein in the absence of either protein suggests that there may be feedback between the two parallel pathways; this would allow partial compensation via the upregulation of the other protein. However, the physical proximity of ER and mitochondria varies from cell type to cell type and is also highly dynamic, which is changing with cellular status (41). Thus, it is possible that the intracellular localization and binding partners of Cisd2 will vary with the cell type and/or with the experimental conditions. This scenario might help to explain why different proteins have been found to interact with Cisd2 under a variety of circumstances by our group and other researchers.

For example, previous studies have demonstrated that Cisd2 forms a complex with Bcl-2 in the ER that is involved in the regulation of autophagy and Ca\(^{2+}\) homeostasis (42). In this study, we provided evidence for the first time to substantiate that, in MAM, Cisd2 in MEFs normally interacts with Gimap5 and thereby these two proteins additively regulate intracellular Ca\(^{2+}\) homeostasis via the promotion of Ca\(^{2+}\) uptake by mitochondria. During the induction of adipogenesis, the expression of Cisd2 was found to be upregulated at an early stage to allow the maintenance of Ca\(^{2+}\) homeostasis and to allow adipogenic signaling to proceed (Fig. 7A). In contrast, a deficiency in Cisd2 was found to cause an elevated level of the cytosolic Ca\(^{2+}\) via an impairment in the Ca\(^{2+}\) buffering capacity of mitochondria; this abnormal elevation of Ca\(^{2+}\) then activates a Ca\(^{2+}\)-calcineurin-dependent pathway that inhibits adipogenic signaling, including the downregulation of various adipogenic genes as well as downregulation of a number of genes related to mitochondrial biogenesis (Fig. 7B). These defects, in turn, affect mitochondrial function, insulin sensitivity and adiponectin secretion in differentiated adipocytes, which may cumulatively result in systemic defects affecting glucose homeostasis in the mouse. The findings of this study enable us to gain a better understanding of the tissue-specific physiological functioning of Cisd2. Furthermore, an amelioration of the age-dependent Cisd2 decline may be able to provide a means of prevention or treatment of metabolic syndrome by way of improving the differentiation and functioning of adipocytes in the human body.

**MATERIALS AND METHODS**

**Generation of adipocyte-specific Cisd2 KO mice**

To generate adipocyte-specific Cisd2 KO mice, mouse Cisd2 genomic DNA was obtained by screening a BAC library (Research Genetics Inc.) derived from the C57BL/6 (B6) mouse strain. A Cisd2 targeting vector, which contains two directly
Figure 7. Cisd2-mediated regulation of white adipogenesis and Ca\(^{2+}\) homeostasis in MEFs. (A) Intracellular Ca\(^{2+}\) homeostasis plays an important role in white adipogenesis. The spatial and temporal activation and/or inhibition of Ca\(^{2+}\)-dependent signaling are highly controlled. Our results revealed that Cisd2 interacts with Gimap5 at the MAM and together these proteins regulate mitochondrial Ca\(^{2+}\) influx and the maintenance of intracellular Ca\(^{2+}\) homeostasis in primary MEFs. In addition, up-regulation of Cisd2 expression at an early stage of adipogenesis positively contributes to the modulation of intracellular Ca\(^{2+}\) and adipogenesis. (B) Cisd2 deficiency impairs mitochondrial Ca\(^{2+}\) influx and decreases the Ca\(^{2+}\) level in mitochondria, which leads to an elevated Ca\(^{2+}\) level in the cytosol. This abnormal Ca\(^{2+}\) level activates calcineurin, which then acts as a negative regulatory effect on white adipogenesis. As a consequence, the induction of adipogenic genes and genes related to mitochondrial biogenesis are compromised during adipogenesis. This scenario may well ultimately cause defects in the functioning of adipocytes, including effects on lipid storage, insulin sensitivity and adiponectin secretion. The end result is likely to culminate in the dysregulation of systemic glucose homeostasis and to manifest clinical features that are known to be associated with metabolic syndrome.

repeats of LoxP sites flanking the promoter and exon 1 of the Cisd2 gene, was then used as homologous recombination arms to create a Cisd2 floxed allele (Cisd2\(^{flx}\)). This Cisd2 targeting vector, which contains the neomycin (Neo) selection cassette flanked with two FRT sites, was linearized and transfected into R1 ES cells. The targeted ES cell clones were screened by Southern blot analysis using 5'-flanking and 3'-flanking probes and the Neo cassette was then eliminated, followed by evaluation by Southern blot analysis using an excision probe. Next, targeted ES cells were injected into B6 blastocysts to produce chimeric
male mice, which were bred with B6 females. Germline transmission was obtained, which was identified via agouti progeny. These heterozygous mice (Cisd2f/+ ) were backcrossed with B6 mice for nine successive generations to introduce the Cisd2 floxed allele (Cisd2f/f ) into the B6 congenic background (Supplementary Material, Fig. S1A–D). The aP2-Cre transgenic mice were purchased from the Jackson Laboratory (JAX 005069) (43) and were then bred with the Cisd2f/f mice. After two generations of breeding, adipocyte-specific Cisd2 KO mice were obtained (Supplementary Material, Fig. S1E). The mice were bred in a specific pathogen-free facility, and the animal protocol was approved by the Institutional Animal Care and User Committee of National Yang-Ming University.

**Total body fat analysis**

Mouse body fat levels were determined using a Micro-CT scanner (SkyScan 1076, Bruker, Kontich, Belgium), and the three-dimensional structure obtained was analyzed to obtain the proportion of fat in the whole body of mice at various ages. The quantitative results were calculated by the software SkyScan 1076.

**Histopathologic examination**

eWAT was collected, fixed with 10% formalin and embedded in the paraffin. Tissue sections (3 μm) were subjected to H&E staining by standard procedures.

**Immunofluorescence staining**

Paraffin-embedded eWAT sections (3 μm) were soaked in an antigen retrieval buffer containing 10 mM sodium citrate (pH 6.0) and heated in a microwave oven for 10 min. This procedure was then repeated once. Next, these cells were fixed using PBS buffer containing paraformaldehyde, 0.4 M sucrose and 2 mM EDTA, for 10 min at room temperature. The fixed cells were permeabilized using 0.2% Triton X-100 and then incubated with 1% BSA at 37°C for 30 min. The tissue sections and pretreated cells were then incubated with specific primary antibodies against perilipin (Cell Signaling Technology, Danvers, MA) and Gimap5 (Novus Biologicals, Littleton, CO) at 4°C for 18–24 h and the signal was detected using a fluorescent secondary antibody (Invitrogen, Eugene, OR). The images were captured by fluorescence microscopy (Olympus BX51, Tokyo, Japan) or confocal microscopy (Zeiss LSM 700, Oberkochen, Germany).

**Measurement of mitochondrial bioenergetic functions using a Seahorse analyzer**

An XF24 analyzer (Seahorse Bioscience, Inc., North Billerica, MA) was used to measure the bioenergetic functioning of mitochondria (46). MEFs and differentiated adipocytes were cultured in a 24-well microplate and the culture medium was replaced by unbuffered DMEM (pH 7.4) at 1 h before measurement. The oxygen consumption rate (OCR) was measured before and after the addition of the indicated chemicals. The OCR was monitored real-time in an incubator chamber at 37°C according to the program provided by the manufacturer and the results are expressed in nmol/min/10^6 cells.

**Transmission electron microscopy**

Mouse tissues were fixed in a mixture of 1.5% glutaraldehyde and 1.5% paraformaldehyde in cacodylate buffer (pH 7.4). They were postfixed in 1% OsO4 and 1.5% potassium hexaferate, then rinsed in cacodylate and 0.2 M sodium maleate buffers (pH 6.0), followed by block staining with 1% uranyl acetate. Following dehydration, the tissues were embedded in Epon and sectioned for transmission electron microscopy (TEM) as described previously (5).

**Measurement of intracellular calcium content**

In order to measure the cytosolic and mitochondrial Ca^{2+} levels, a single coverslip of cells was stained with 5 μM Fura-2/AM or with Rhod-2/AM in buffer (150 mM NaCl, 5 mM glucose, 1 mM Ca^{2+}, 1 mM Mg^{2+}, 5 mM KCl, 10 mM HEPES (pH 7.4), 0.5% BSA) for 30 min. The cells were then washed 3× with warm buffer and equilibrated for 30 min before measurement. The fluorescence signal of each cell was monitored real-time using a fluorescence microscope (Zeiss Axio Observer Z1) equipped with a spectrofluorimeter (OM Ni-CCD 544 plus, Andor Technology) and a Zyla 5.5 sCMOS camera (Andor Technology) controlled by a computer using Andor Omega software (v 1.5). The absorbance was expressed as arbitrary fluorescence units (AFUs).

**Glucose uptake assay**

Glucose uptake was measured according to a method described previously (45). The adipocytes were stimulated with (insulin-stimulated group) or without (basal group) 100 nM insulin for 18–24 h and the conditioned medium was collected and centrifuged to remove the cells and cell debris. The concentration of adiponectin was measured using an ELISA kit (Merck Millipore, Billerica, MA) according to the manufacturer’s instructions.
Measurement of intracellular calcium content using aequorin
The double-mutated form (Asp119Ala and Asn285leu) of AEQ was used to measure the concentration of Ca^{2+} in the millimolar range for a long period of time. The mitochondria-targeted aequorin (mtAEQ) contains the mitochondrial presequence of subunit IV of cytochrome c oxidase. The ER-targeted aequorin (erAEQ) contains the ER leading peptide sequence, namely the VDJ and CH1 domains of an IgG2b heavy chain. Cells were transfected with either mtAEQ or erAEQ and were reconstituted with 5 \(\mu\)M coelenterazine for 45 min at 37°C in the KRB buffer (135 mM NaCl, 5 mM KCl, 0.4 mM KH2PO4, 1 mM MgSO4, 5.5 mM glucose, 20 mM HEPES, pH 7.4) supplemented with 1 mM CaCl2. After the cells had been washed, luminescence intensity was measured using an Infinite 200 Microplate Reader (Tecan Group Ltd., Mannending, Switzerland) (27).

Calcineurin activity assay
Excess phosphate and nucleotides in the cell lysate were first removed using a desalting column. Total serine/threonine phosphatase activity was then determined by incubating the cell lysate with RII substrate (Enzo Life Sciences, Farmingdale, NY) for 20 min at 30°C in 50 mM Tris–HCl buffer (pH 7.0) containing 100 mM NaCl, 6 mM MgCl2, 0.5 mM DTT and 0.5 mM CaCl2. The released phosphate was detected using a spectrophotometer. The activity was calculated by subtracting the activity in the presence of EGTA from the total calcineurin activity.

Split YFP assay
cDNA encoding the C-terminus of Venus protein (VC) was introduced before 5′ and after 3′ the open reading frame of Cisd2 in order to generate two fusion proteins, VC-Cisd2 and Cisd2-VC, respectively. Similarly, cDNA encoding the N-terminus of the Venus protein (VN) was introduced before 5′ and after 3′ of open reading frame of Gimap5 in order to generate two fusion proteins, VN-Gimap5 and Gimap5-VN, respectively. Different combinations of the constructs (1 \(\mu\)g) were then cotransfected into HEK-293T cells for 48 h, and fluorescence images of the cells captured using a confocal microscope (Zeiss LSM 700) in order to monitor directly the interaction between Cisd2 and Gimap5.

Isolation of MAM
The protocol established by Wieckowski et al. (48) was employed to isolate the MAM from MEFs. Briefly, after cells had been homogenized (H) and the nuclei and cell debris were removed by low-speed centrifugation (800g), crude cytosolic (Cc) and crude mitochondrial (Mc) fractions were obtained by high-speed centrifugation (10000g) of the homogenate as the supernatant and pellet, respectively. The supernatant and pellet, respectively. The crude mitochondrial fraction was further centrifuged at 100000g for 1 h to divide it into a pure cytosolic fraction (Cp) and a microsome fraction (ER) as the supernatant and pellet, respectively. In addition, the crude mitochondrial fraction was placed on the top of a 30% Percoll medium and centrifuged at 95000g using a swing bucket rotor. After 30 min, the MAM and mitochondria (Mp) were localized as upper and lower visible bands in the Percoll medium, respectively. All of the above procedures were carried out at 4°C to prevent protein degradation.

Silver staining
SDS–PAGE gels were fixed overnight in a solution containing 40% methanol and 10% acetic acid, which was then replaced with 30% methanol solution for 15 min. After washing with deionized H2O, the gel was sensitized by adding 0.02% sodium thiosulfate for 2 min and then stained with 0.2% silver nitrate for 30 min in the dark. A developing solution containing 3% sodium carbonate and 0.05 ml/dl formaldehyde was added next; finally, a 1.4% EDTA solution was used to stop the reaction when protein bands had become visible.

Immunoprecipitation assay
GFP-Cisd2 and endogenous Gimap5 were purified from the total lysate of HEK-293T cells and MEFs using a Mag Sepharose Xtra kit (GE Healthcare Life Sciences, Buckinghamshire, UK) according to the manufacturer’s instructions. In outline, 1 mg total protein lysate of cells was incubated with specific primary antibodies at 4°C overnight, which was followed by incubating the mixture with 20 \(\mu\)l of immunocapture beads at 4°C for 4 h. After washing with TBST buffer (50 mM Tris, 150 mM NaCl and 0.1% Tween-20, pH 7.4), the target protein and its interacting proteins were eluted by 2% SDS and were analyzed by western blotting.

Lentiviral knockdown of desired genes with shRNA
Small hairpin RNAs (shRNAs) were designed, respectively, for Cisd2 gene (shCisd2), Gimap5 gene (shGimap5) and the luciferase gene (shLuc), which served as a scramble control. All of the shRNAs were introduced into the expression plasmid pLKO.1 and were packaged in lentiviruses from the RNAi Core Facility at Academia Sinica, Taipei, Taiwan. The target sequences were as follows: shLuc, 5′-CGTGGAGTATATTGATCCAG-3′; shCisd2 #1 and #2, 5′-GCACAGAAGGATA GCTA-3′ and 5′-CCCAAGGTGTGAA TGGAGA-3′, respectively; shGimap5 #1 and #2, 5′-CTTGTGTATTTAGCGGT TAA-3′ and 5′-GTTTGACTTCCTTCA CCCACTT-3′, respectively.
Statistical analysis
Statistical analyses were performed using the Microsoft Excel 2007 statistical package and the date are presented as means ± SD of the results obtained from three or more independent experiments. The significance level of the difference between control and experimental groups was determined by Student's t test. A difference is considered significant when *, P < 0.05 or **, P < 0.005.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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