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ORIGINAL RESEARCH ARTICLE

Ago2 Protects Against Diabetic Cardiomyopathy by Activating Mitochondrial Gene Translation

Jiabing Zhan*; Kunying Jin*; Rong Xie*; Jiahui Fan; Yuyan Tang; Chen Chen†; Huaping Li†; Dao Wen Wang†

BACKGROUND: Diabetes is associated with cardiovascular complications. microRNAs translocate into subcellular organelles to modify genes involved in diabetic cardiomyopathy. However, functional properties of subcellular Ago2 (Argonaute2), a core member of miRNA machinery, remain elusive.

METHODS: We elucidated the function and mechanism of subcellular localized Ago2 on mouse models for diabetes and diabetic cardiomyopathy. Recombinant adeno-associated virus type 9 was used to deliver Ago2 to mice through the tail vein. Cardiac structure and functions were assessed by echocardiography and catheter manometer system.

RESULTS: Ago2 was decreased in mitochondria of diabetic cardiomyocytes. Overexpression of mitochondrial Ago2 attenuated diabetes-induced cardiac dysfunction. Ago2 recruited *TUFM*, a mitochondria translation elongation factor, to activate translation of electron transport chain subunits and decrease reactive oxygen species. Malonylation, a posttranslational modification of Ago2, reduced the importing of Ago2 into mitochondria in diabetic cardiomyopathy. Ago2 malonylation was regulated by a cytoplasmic-localized short isoform of *SIRT3* through a previously unknown demalonylase function.

CONCLUSIONS: Our findings reveal that the *SIRT3*–Ago2–*CYTB* axis links glucotoxicity to cardiac electron transport chain imbalance, providing new mechanistic insights and the basis to develop mitochondria targeting therapies for diabetic cardiomyopathy.

Key Words: diabetic cardiomyopathies ■ mitochondria ■ therapeutics

Global diabetes prevalence in 2019 was estimated to be 9.3% (463 million people), and is predicted to rise to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045.[1] Cardiovascular complications account for >80% of deaths resulting from diabetes.[2] The need for an absence of coronary artery disease, hypertension, or any other form of cardiac disease to diagnose diabetic cardiomyopathy is unrealistic because diabetes is a common comorbidity with other cardiac diseases.[3,4] It has been suggested that diabetic cardiomyopathy should be defined as "cardiac abnormalities not wholly explained by other cardiovascular or noncardiovascular causes and likely to be due to diabetes."[5] In terms of treatment, although the sodium-glucose cotransporter 2 inhibitor–containing anti–heart failure (HF) therapies reduce the risk of cardiovascular death or hospitalized HF in patients with type 2 diabetes,[6] the risk of dying from diabetes-related HF remains high.[7] Better understanding of the mechanisms underlying diabetic cardiomyopathy is needed to develop new intervention strategies.

Diabetic cardiomyopathy may involve complex mechanisms, including glucose toxicity, lipotoxicity, elevated apoptotic and necrotic cell death, impaired Ca^{2+} balance, cross-linking of advanced glycation end products (AGEs), mitochondrial damage, oxidative stress, and inflammation.[4] Mitochondria are the center of metabolism, and recent data suggest that mitochondrial dysfunction

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Clinical Perspective

What Is New?

- Decreased mitochondrial Ago2 (Argonaute2)–CYTB (cytochrome B) axis links glucotoxicity to electron transport chain imbalance and oxidative stress in the heart, which provides new insights for diabetic cardiomyopathy.
- Mitochondrial Ago2/microRNAs directly interact with translation elongation factor TUFM (Tu translation elongation factor) to enhance mitochondrial translation.
- Loss of a cytoplasmic short form of SIRT3 (sirtuin 3) in diabetic heart leads to increased Ago2 malonylation, which is responsible for compromised Ago2 transport into mitochondria by Timm17b-containing transporter.

What Are the Clinical Implications?

- Recombinant adeno-associated virus type 9–mediated mitochondrial Ago2 delivery rescues cardiac dysfunction in diabetic models.
- In the human failing heart in diabetes, mitochondrial Ago2 is decreased, supporting its potential as a novel therapeutic target for patients with diabetes-induced cardiac dysfunction.

directly contributes to oxidative stress and cell death, which promotes the progression of diabetic cardiomyopathy.[8–10] Mitochondrial electron transport chain (ETC) complexes are the major source of mitochondrial reactive oxygen species (mt-ROS).[11,12] A recent study observed that upregulation or downregulation of the same ETC subunit similarly augmented reactive oxygen species (ROS), indicating that mitochondrial imbalance was the cause, rather than the altered function of the specific genes involved, for elevated ROS production in neuronal cells.[13] However, the mechanism underlying mitochondrial imbalance in diabetic cardiomyopathy is largely unknown.

By focusing on diabetic cardiomyopathy–related microRNA (miRNA), we have uncovered the functional properties of many subcellular localized miRNAs.[12,14–17] Of note, we reported that in diabetes, cardiac miRNAs translocated into mitochondria or the nucleus to enhance mitochondria-encoded gene translation or nucleus-encoded gene transcription in a noncanonical fashion.[12,14,15] However, for clinical practice, targeting subcellular localized miRNAs is difficult because of the lack of well-established subcellular localization sequences for miRNAs. We found that Ago2 (Argonaute2), a core component of the miRNA-RISC (miRNA-induced silencing complex) machinery, is presented in subcellular fractions.[12] The localization of Ago2 in subcellular fractions was also reported by other studies,[18–21] indicating that subcellular Ago2 might be critical for preserving the function of subcellular organelles. Moreover, Ago2 appears to undergo posttranslational modifications (PTMs), with several studies demonstrating that phosphorylation of Ago2 affects its cytoplasmic localization and granular distribution in HEK293 cells.[22,23] However, the roles of Ago2 in diabetic cardiomyopathy and the mechanism underlying subcellular Ago2 localization or Ago2 PTMs in myocytes remain unclear.

Nonstandard Abbreviations and Acronyms

AGE	advanced glycation end product
Ago2	Argonaute2
CDS	coding sequence
CO2	cytochrome c oxidase 2
COX8	cytochrome c oxidase subunit 8
CYTB	cytochrome B
ETC	electron transport chain
GFM1	G elongation factor mitochondrial 1
HF	heart failure
KO	knockout
MID	middle
miRNA	microRNA
miRNA-RISC	microRNA-induced silencing complex
MITRAC	mitochondrial respiratory-chain complex
MS	mass spectrometry
mt-ROS	mitochondrial reactive oxygen species
MTIF2	mitochondrial translational initiation factor 2
MTIF3	mitochondrial translational initiation factor 3
NAD	nicotinamide adenine dinucleotide
ND4	NADH dehydrogenase 4
NES	nuclear export signal
NLS	nuclear localization signal
PIWI	P-element-induced wimpy testis
PRIDE	Proteomics Identification Database
PTM	posttranslational modification
rAAV	recombinant adeno-associated virus
ROS	reactive oxygen species
SIRT5	sirtuin 5
TACO1	translational activator of cytochrome c oxidase I
TPM	transcripts per million reads
TUFM	Tu translation elongation factor
VDAC1	voltage-dependent anion-selective channel 1

In this study, we found that subcellular Ago2 was rearranged in the failing heart of mice and patients with diabetes. Diabetes induced Ago2 malonylation, a new form of PTM, which directly impaired Ago2 translocation into mitochondria, leading to mitochondrial translation suppression, imbalance of ETC complexes, and oxidative stress. Ago2 with a specific mitochondrial localization signal (but not a nuclear or cytoplasm signal) delivered by recombinant adeno-associated virus (rAAV) was able to rescue the cardiac dysfunction in mice with diabetes, suggesting a potential therapy for diabetic cardiomyopathy.

METHODS

Detailed methods are provided in the Supplemental Material. We deposited the RNA sequencing data to Gene Expression Omnibus (accession numbers GSE224684, GSE241907, GSE241911, GSE242024, GSE242088, and GSE242185). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium using the PRIDE (Proteomics Identification Database) partner repository (data set identifier PXD044944). Data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval

All animal studies were conducted with the approval of the animal research committee of Tongji Medical College and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Human heart tissues were collected according to a protocol approved by the Clinical Research Committee of Tongji Medical College (Wuhan, China). The study also conformed to the principles outlined in the Declaration of Helsinki.

Statistical Analysis

Data are presented as mean±SEM; n is noted in specific figure legends. All data sets were tested for normality using the Shapiro-Wilk test to select an appropriate parametric or nonparametric test. Student *t* tests were performed to determine statistical significance between 2 groups. One-way ANOVA combined with Tukey multiple comparisons was performed for comparisons among >2 groups. Wilcoxon signed-rank tests were applied for nonparametric paired analysis (2 groups). In all cases, statistical significance was defined as $P<0.05$.

RESULTS

Downregulation of Ago2 in Cardiomyocyte Mitochondria Under Diabetic Conditions

Streptozotocin-treated mice and leptin receptor–deficient db/db mice are both well-established rodent models for type 1 and type 2 diabetes and diabetic complications.[24–26] Our previous studies have revealed that type 1 diabetic mice at 8 weeks after streptozotocin treatment and type 2 diabetic db/db mice at 16 weeks of age developed cardiac dysfunction.[12,17] To determine the causal events in diabetic cardiomyopathy rather than the consequential changes of decreased cardiac performance, we chose to evaluate Ago2 subcellular localization in mice at 4 weeks after streptozotocin treatment and in db/db mice at 8 weeks of age; these time points preceded the decrease of cardiac dysfunction. Under these conditions, we selectively enriched for subcellular fractions by differential sedimentation (nucleus and cytoplasm isolation) or anti-TOM22 antibody–conjugated magnetic beads (mitochondria isolation). The isolated nucleus was free from contamination, as indicated by the lack of cytosolic protein GAPDH and mitochondrial protein VDAC1 (voltage-dependent anion-selective channel 1), whereas the mitochondrial fraction was largely free of nuclear-specific protein LaminB and cytosolic GAPDH (Figure 1A). Using these highly purified components, we found that in type 1 diabetic mice, cardiac Ago2 was decreased in the nucleus and mitochondria; however, cytoplasm-localized Ago2 was unchanged (Figure 1B and 1C). Type 2 diabetic mice showed decreased Ago2 localization in mitochondria compared with unaltered expression in other subcellular fractions (Figure 1D and 1E). Using immunofluorescence, we also observed decreased colocalization of Ago2 with HSP60 (mitochondrial marker) in myocytes isolated from 2 types of diabetic mouse heart (Figure S1A and S1B). A downregulation of mitochondrial Ago2 was also observed in human patients with HF with diabetes in comparison with patients with HF without diabetes. (Figure S1C). Because the cytoplasmic form of Ago2 was unchanged in both type 1 and type 2 diabetic mice (Figure 1C and 1E), decreased localization of Ago2 in mitochondria appears to be attributable to impaired translocation from cytoplasm into mitochondria in these diabetic models.

Mitochondrial Ago2 Protected Against Cardiac Dysfunction in Diabetic Mice

To investigate the functional relevance of Ago2 in diabetes-induced cardiac dysfunction, we performed gain-of-function and loss-of-function experiments. Ago2 was predominantly expressed in myocytes, as suggested by the published single-cell sequencing data[27] and the Human Protein Atlas database.[28] We therefore adopted the rAAV9 system under a troponin T (tnt) promoter (rAAV-tnt) to achieve cardiomyocyte-specific Ago2 expression. We engineered the vectors expressing the nuclear, cytosolic, and mitochondrial forms of Ago2 by fusing it to a nuclear localization signal (NLS), nuclear export signal (NES), or the cytochrome c oxidase subunit 8 (COX8) mitochondria targeting sequence (Figure 2A; Figure S2A). Using these subcellular-specific Ago2 vectors, we found that the mitochondrial form of Ago2 overexpression protected against cardiac dysfunction and attenuated cardiac hypertrophy in streptozotocin-treated

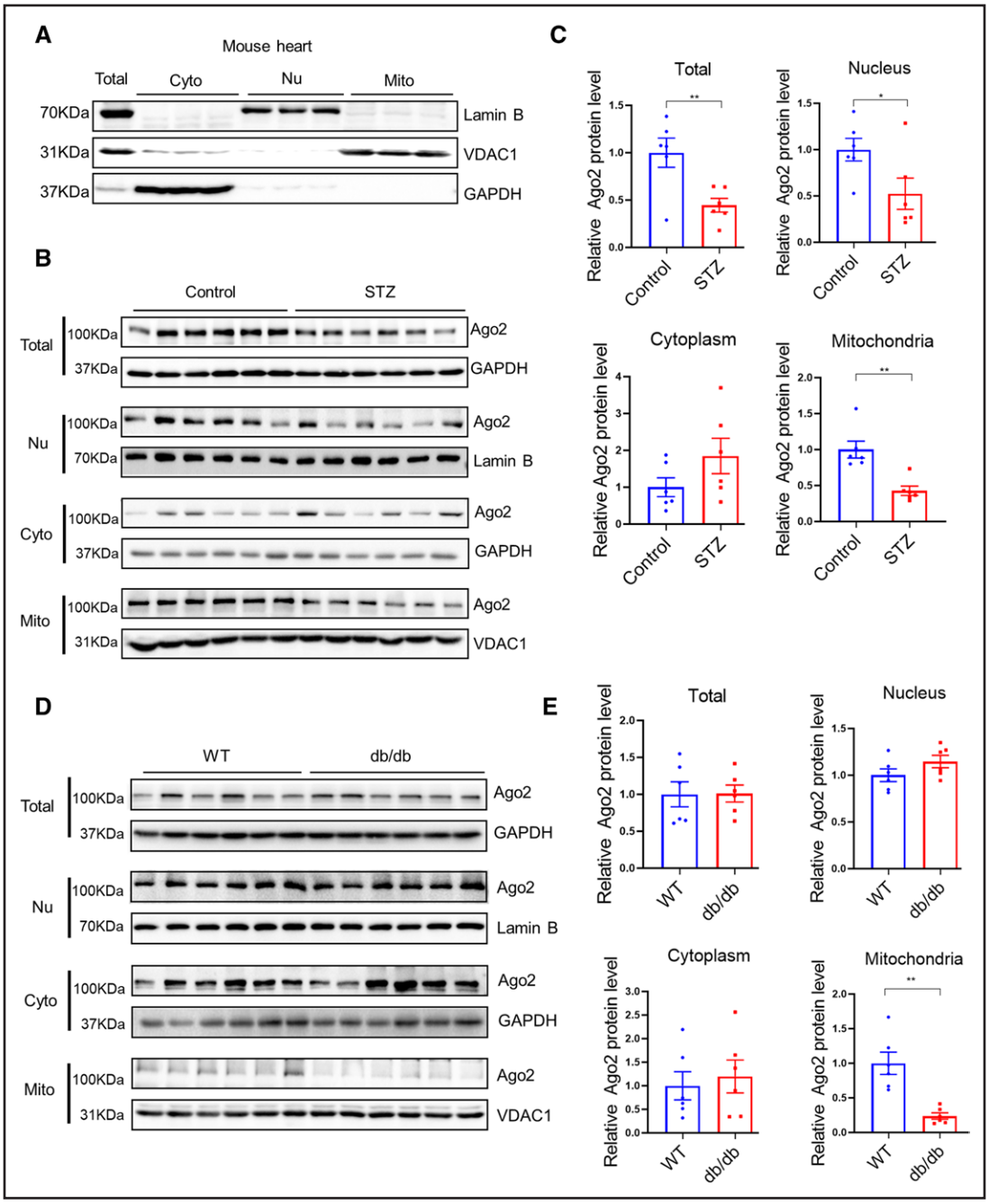

Figure 1. Downregulation of Ago2 in mitochondria of cardiomyocytes under diabetic conditions.
A, Western blot analysis of subcellular fractions from mouse heart. Antibodies against Lamin B, VDAC1 (voltage-dependent anion-selective channel 1), and GAPDH were used to determine the purity of nuclear, mitochondrial, and cytosol fractions, respectively. **B** and **C**, Western blot analysis of Ago2 (Argonaute2) levels in different subcellular fractions from hearts of streptozotocin (STZ)–induced diabetic mice (n = 6). **D** and **E**, Western blot analysis of Ago2 levels in different subcellular fractions from hearts of db/db mice (n=6). **B** through **E**, **P*<0.05, ***P*<0.01; Student *t* test. WT indicates wild type.

mice (Figure 2B through 2D; Table S1; Figure S2B and S2C). Even after the onset of cardiac dysfunction, mitochondrial Ago2 overexpression was able to improve cardiac performance (Figure S3A through S3C). In sharp contrast, neither the cytosolic nor nuclear form of Ago2 showed any detectable effects (Figure 2B through 2D; Table S1), despite successful induction of Ago2 in these fractions (Figure S4A through S4E).

We then performed a reciprocal study by knockdown Ago2 in the heart using shRNA delivered by rAAV9. As expected, rAAV9-tnt-Ago2-shRNA decreased cardiac Ago2 levels (Figure S5A). Cardiac function was not affected by Ago2 knockdown (Figure S5B through S5D; Table S2). We reasoned that because mitochondrial Ago2 already was impaired in streptozotocin-treated mice, further decreasing the Ago2 level may not have

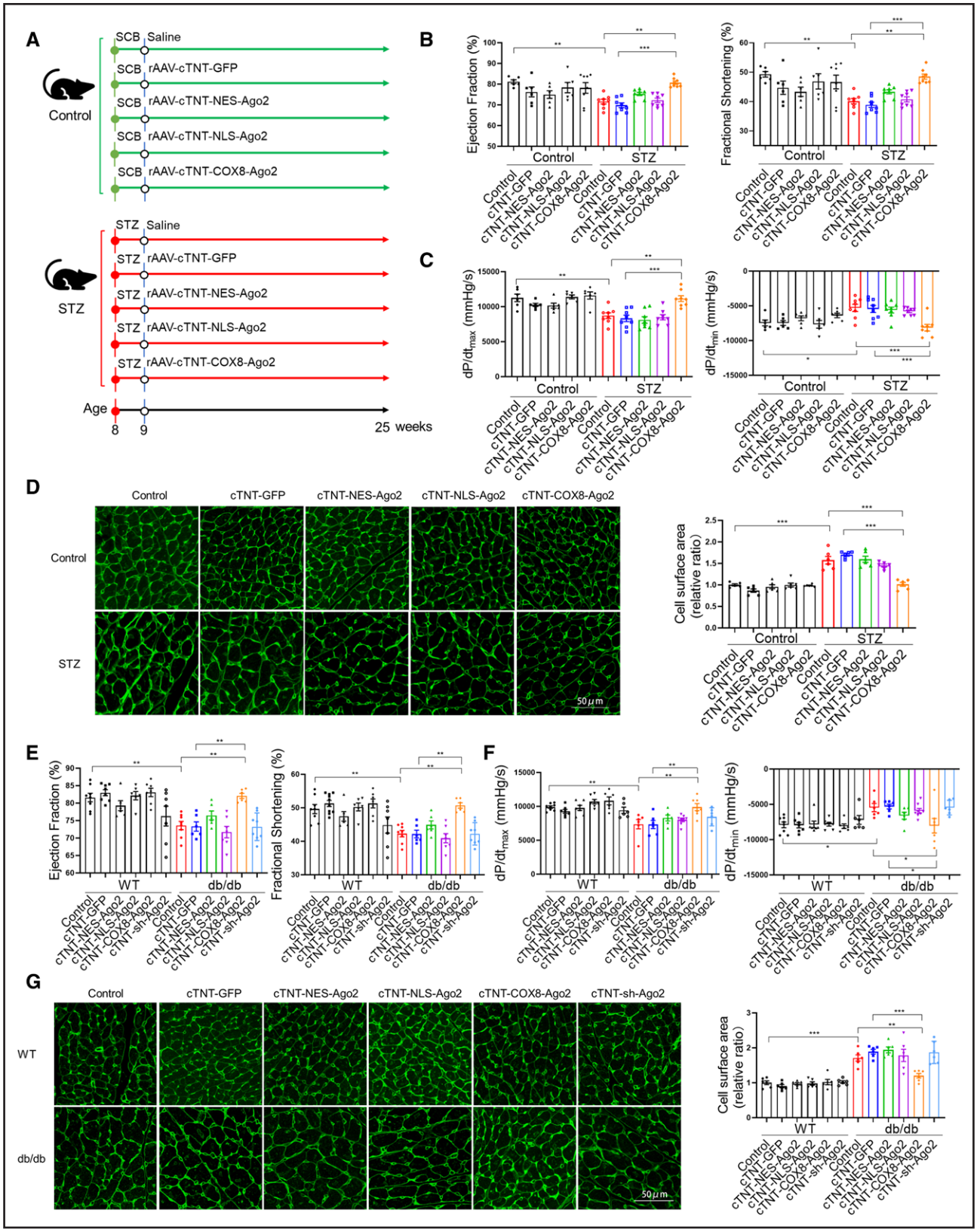

Figure 2. Mitochondrial Ago2 protected against cardiac dysfunction in diabetic mice.

A, Animal strategy for studying the effect of subcellular Ago2 (Argonaute2) overexpression on the cardiac function of streptozotocin (STZ)–induced diabetic mice (n=6–8). **B** and **C**, Echocardiographic and hemodynamic analysis in different groups of streptozotocin-induced diabetic mice (n=6–8). **D**, Representative images of wheat germ agglutinin staining on cardiac sections and quantitative analysis in different groups of streptozotocin-induced diabetic mice (n=5 or 6). **E** and **F**, Echocardiographic and hemodynamic analysis in different groups of db/db mice (n=6–8). **G**, Representative images of wheat germ agglutinin staining on cardiac sections and quantitative analysis in different groups of (*Continued*)

Figure 2 Continued. db/db mice (n=5 or 6). **B** through **G**, *P<0.05, **P<0.01, ***P<0.001; 1-way ANOVA followed by Tukey multiple comparisons; number of comparisons=45 (for **B** through **D**) or 66 (for **E** through **G**). All P values were adjusted for multiple comparisons, and the comparisons with pathophysiologic relevance were marked out; the same is true for subsequent figures when multiple comparisons were performed. COX8 indicates mitochondrial localization cox8 signal; dp/dt_{max}, peak instantaneous rate of left ventricular pressure increase; dp/dt_{min}, peak instantaneous rate of left ventricular pressure decrease; NES, nuclear export signal; NLS, nuclear localization signal; SCB, sodium citrate buffer; and WT, wild type.

not affected cardiac performance, or Ago2 knockdown decreased nuclear and cytoplasmic Ago2, which might interfere with the effects mediated by mitochondria. Consistent with the results obtained from the streptozotocin model, a similar regulation pattern was also observed in the type 2 db/db diabetic model under similar treatments (Figure 2E through 2G; Figure S6; Table S3). No significant difference in cardiac function or cardiac hypertrophy was found under Ago2 overexpression in the normal control mice (Figure 2B through 2G), indicating regulation of Ago2 in the heart only under stress conditions.

Mitochondrial Ago2 Enhanced Mitochondrial Gene Expression to Quench ROS Production

Considering that only mitochondrial Ago2 overexpression improved cardiac function in diabetic mice, we first performed mass spectrometry (MS) analysis on the hearts from streptozotocin-treated mice to specifically evaluate mitochondrial gene-encoded proteins, which appeared to be directly regulated by the Ago2/miRNA complex by a translational mechanism.[20,29] We noticed that subunits in ETC I (ND2/ND4/ND5) and ETC III (CYTB) were significantly decreased, whereas the subunits in ETC IV (COI/II/III) and ETC V (such as ATP6 and ATP8) were relatively less changed (Figure 3A; Excel S1), indicating mitochondrial imbalance under diabetic conditions. Meanwhile, several pathways, such as fatty acids metabolism, PPAR signaling, and peroxisome, were significantly dysregulated in the streptozotocin hearts (Figure S7A; Excel S1), which has been extensively studied and reviewed.[30,31] However, considering their cytoplasmic localization, these significantly dysregulated pathways were unlikely to be the direct downstream targets for mitochondrial Ago2, and were not studied further.

MS technology is not absolutely quantitative because peptide response depends on sample matrix as well as ionization efficiencies, which are nonlinear responses and may not accurately reflect the real fold change. We therefore performed Western blotting validation on representative subunits of ETCs. Largely consistent with the regulation trends observed from MS data, we found that the representative subunits ND4 (NADH dehydrogenase 4; ETC I) and CYTB (cytochrome B; ETC III) were dramatically decreased; CO2 (cytochrome c oxidase 2; ETC IV) and ATP6 (ETC V) were less changed (Figure 3B).

According to our previous studies, knockdown of *CYTB*, the only mitochondrial gene-encoded subunit of ETC III, significantly activated mitochondrial ROS production compared with other ETCs[14,15]; this is consistent with the results of other studies showing that inhibition of ETC III is more potent than that of other ETCs in inducing ROS production.[32,33] We thus particularly focused on the function of *CYTB*-mediated ROS regulation in the context of diabetes-induced cardiac dysfunction. Under mitochondrial Ago2 overexpression conditions, CYTB of ETC III was significantly increased and CO2 (ETC IV) and ATP6 (ETC V) were relatively unchanged in the heart of streptozotocin-treated mice (Figure 3C and 3D). Moreover, Ago2 overexpression also protected against unbalanced decrease of respiratory chain supercomplexes assembly in streptozotocin-treated mice (Figure S7B through S7D). To test whether loss of Ago2 would decrease *CYTB* expression, we performed ribosome profiling sequencing to evaluate the whole translatomics in Ago2 knockout (KO) myocytes (AC16 cell). Several nuclear-encoded subunits were upregulated, whereas most mitochondrial gene-encoded subunits were downregulated, by Ago2 KO, among which CYTB decreased most dramatically (Figure S7E; Excel S2 and S3), which further indicated the unbalanced regulation of Ago2 on ETCs. Most downregulated mt-ETCs mediated by Ago2 KO were rescued by mito-Ago2 to some extent, except for a few subunits, especially mt-ATP6. Translation of mt-ATP6 and mt-CO2 were downregulated in Ago2 KO myocyte (human AC16), but failed to be upregulated by mito-Ago2 overexpression in mouse heart (Figure 3C and Excel S4 and S5), which might be attributable to interspecies differences in nucleotide sequence of mt-genes, or because total Ago2 KO was not completely equal to loss of mitochondrial Ago2. In contrast to ATP6 and CO2, the regulation of mt-CYTB by Ago2 was coordinated among different species and between in vivo and in vitro studies, indicating a conserved pathophysiologic role of mt-CYTB.

Because decreased CYTB directly contributed to ROS production, we measured ROS levels in diabetic mice, observing that mitochondrial Ago2 overexpression decreased streptozotocin-induced ROS activation (Figure 3E; Figure S8A through S8C). In the db/db type 2 diabetic mouse hearts, we also observed restored *CYTB* expression and attenuated ROS level by mitochondrial Ago2 treatment (Figure S8D and S8E). In addition to ROS, abnormalities in mitochondrial energy production (ATP levels) as well as mitochondrial respiration were also reversed by mitochondrial Ago2 treatment (Figure S8F and S8G).

We then attempted to determine whether mitochondrial Ago2 decreased the ROS level by restoring CYTB

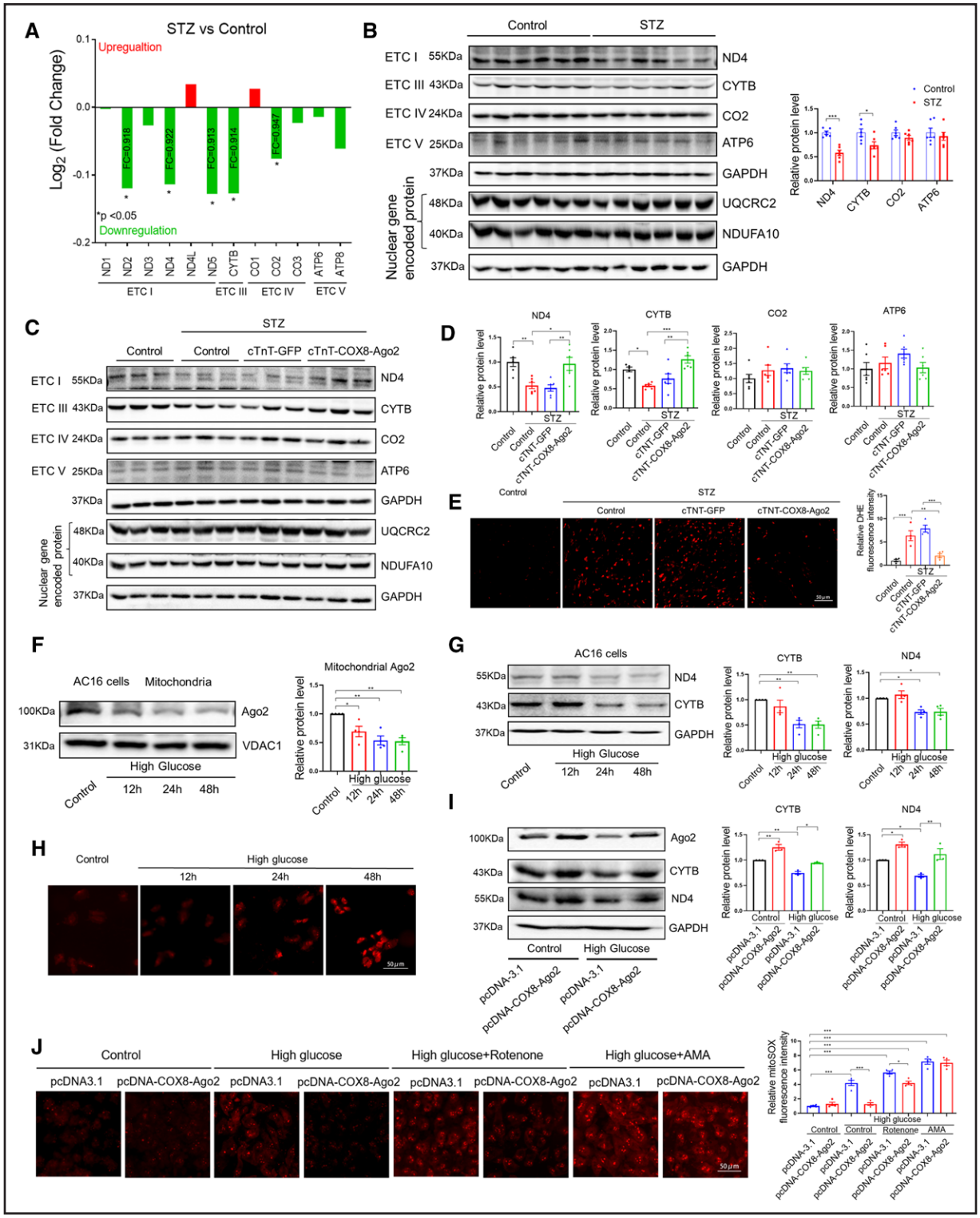

Figure 3. Mitochondrial Ago2 enhanced mitochondrial gene expression to quench ROS production.
A, Mitochondrial gene-encoded protein levels in streptozotocin (STZ)–induced diabetic mice on the basis of data from mass spectrometry analysis (n=7). **B**, Western blot analysis of cardiac mitochondrial gene-encoded proteins and nuclear gene-encoded proteins in streptozotocin-induced diabetic mice (n=6). **C** and **D**, Western blot analysis of cardiac mitochondrial gene-encoded proteins and nuclear gene-encoded proteins in streptozotocin-induced diabetic mice overexpressing cardiac mitochondria-located Ago2 (Argonaute2; n=5 or 6). **E**, Detection of cardiac reactive oxygen species (ROS) by DHE staining in streptozotocin-induced diabetic mice overexpressing cardiac mitochondrial Ago2 (n=4). **F**, Western blot analysis of mitochondrial Ago2 in AC16 cells treated with high glucose (33.3 mM) for 12, 24, and 48 hours (n=4). **G**, Western blot analysis of ND4 (NADH dehydrogenase 4) and CYTB (cytochrome B) in AC16 cells treated with high glucose (33.3 mM) for 12, 24, and 48 hours (n=4). **H**, Detection of mitochondrial superoxide using MitoSox in AC16 cells treated with high glucose (33.3 mM) for 12, 24, and 48 hours. **I**, Western blot analysis of ND4 and CYTB in AC16 cells overexpressing mitochondrial Ago2 under control (5 mM) or high glucose (33.3 mM) (*Continued*)

Figure 3 Continued. conditions (n=3). **J**, Detection of mitochondrial superoxide using MitoSox in AC16 cells treated with AMA (antimycin A)/rotenone and mitochondrial Ago2 overexpression under control (5 mM) or high glucose (33.3 mM) conditions (n=4). **B** through **J**, *P<0.05, **P<0.01, ***P<0.001. **B**, Student t test. **D** through **G**, **I**, and **J**, 1-way ANOVA followed by Tukey multiple comparisons; number of comparisons=6 (for **D** through **G** and **I**) or 28 (for **J**). ETC indicates electron transport chain.

expression under hyperglycemia in vitro. In cultured myocytes, we observed decrease of Ago2, downregulation of CYTB, and upregulation of ROS by high glucose treatment (Figure 3F through 3H). Mitochondrial Ago2 overexpression increased the CYTB level and suppressed ROS production (Figure 3I and 3J; Figure S9A through S9C). The regulation of mito-Ago2 on ROS was independent of osmolarity (Figure S9D and S9E). Moreover, only mito-Ago2 (but not nuclear or cytosol Ago2) was able to decrease ROS induced by hyperglycemia (Figure S9F). Considering that CYTB is a subunit of ETC III, we also performed an ETC III blockage assay, observing that mt-ROS could also be induced by antimycin A (inhibitor of electron transfer at ETC III), and, to a lesser extent, by rotenone (inhibitor of electron transfer at ETC I; Figure S9G). Mito-Ago2 was unable to attenuate mt-ROS in cells treated with antimycin A, but could partially attenuate mt-ROS in cells treated with rotenone under high glucose conditions (Figure 3J). These data indicated that the regulation of ROS conferred by mito-Ago2 is mainly dependent on electron transfer at ETC III.

Mitochondrial Ago2 Enhanced Mitochondrial Gene Translation by Recruiting TUFM

Having revealed the downstream targeting gene of mitochondrial Ago2, we then investigated the detailed mechanism underlying mitochondrial Ago2-induced *CYTB* overexpression. The mRNAs of all mitochondria gene-encoded subunits, including *CYTB*, were unaffected by mitochondrial Ago2, indicating a post-transcriptional regulation patten (Figure S10A and S10B). We therefore evaluated Ago2-mediated translation activation in mitochondria. Proteins involved in mitochondrial translation included MTIF2 (mitochondrial translational initiation factor 2), MTIF3 (mitochondrial translational initiation factor 3), MITRAC (mitochondrial respiratory-chain complex), TACO1 (translational activator of cytochrome c oxidase I), and C12orf62 for translation initiation, and TUFM (Tu translation elongation factor) and elongation factor Ts and GFM1 (G elongation factor mitochondrial 1) for translation elongation.[34–36] Using highly purified mitochondria, we performed Ago2 immunoprecipitation followed by MS analysis of associated proteins, finding that TUFM and TACO1 was captured by Ago2 antibody relative to anti–immunoglobulin G control (Figure 4A; Excel S6; Figure S10C and S10D). The association of TUFM with Ago2 was validated reciprocally by coimmunoprecipitation using a TUFM antibody (Figure 4A). As for TACO1, a specific translation activator for mt-CO1 was also associated with Ago2 (Figure S10D); however, it was unable to explain increased translation of mt-CYTB and therefore was not selected. Moreover, transcriptional factors such as TEFM were also associated with Ago2; however, this is unlikely to mediate the upregulation of mt-genes by mito-Ago2 because steady levels of mt-RNAs were unaffected (Figure S10A and S10B).

We performed immunoprecipitation experiments using truncated fragment of Flag-tagged Ago2, finding that the MID (middle) and PIWI (P-element-induced wimpy testis) domains of Ago2 were able to be captured by TUFM protein (Figure S10E). Furthermore, a loss-of-function study showed that Ago2 knockdown decreased TUFM association with mt-mRNAs (CYTB and ND4), whereas TUFM knockdown had no effects on Ago2 binding events to mt-mRNAs (Figure 4B and 4C). These data suggested that mitochondrial Ago2 increases the recruitment of TUFM to mt-mRNAs, but not vice versa. Furthermore, to test whether Ago2-mediated TUFM mt-mRNA associations are functional, we used a TUFM siRNA to mimic TUFM detachment from mt-mRNAs induced by Ago2 knockdown. We found that TUFM knockdown led to decreased expression of mitochondrial encoded subunits CYTB and ND4 (Figure 4D). Moreover, mitochondrial Ago2 overexpression-induced CYTB expression was abolished by TUFM knockdown (Figure 4E). These data indicate that decreased Ago2 in the diabetic heart might contribute to detachment of TUFM from mt-mRNAs, further leading to the impaired translation of mitochondrial genes.

Participation of Mitochondrial miRNAs in Mitochondrial Ago2 Loss-Mediated ETC Imbalance

If Ago2 loss-mediated TUFM detachment from mt-mRNAs was indeed the cause of decreased mitochondrial subunits, then all mitochondrial genes should be equally decreased in the diabetic heart and rescued by mitochondrial Ago2 overexpression because TUFM is a translation regulator for all mitochondrial genes. This selective regulation pattern of Ago2 prompted us to test whether miRNAs were involved in such mitochondrial Ago2-mediated translational control, because miRNAs usually exerted their function through base-paring rules with high sequence specificity. To this end, we performed miRNA sequencing in magnetic beads–purified mitochondria from control and diabetic hearts (each group was pooled from 3 mouse samples). As a result, 543 miRNAs were identified in isolated mitochondria, 66 of which were selected for further study on the basis of a TPM (transcripts per million reads) cutoff of 1000 (Excel

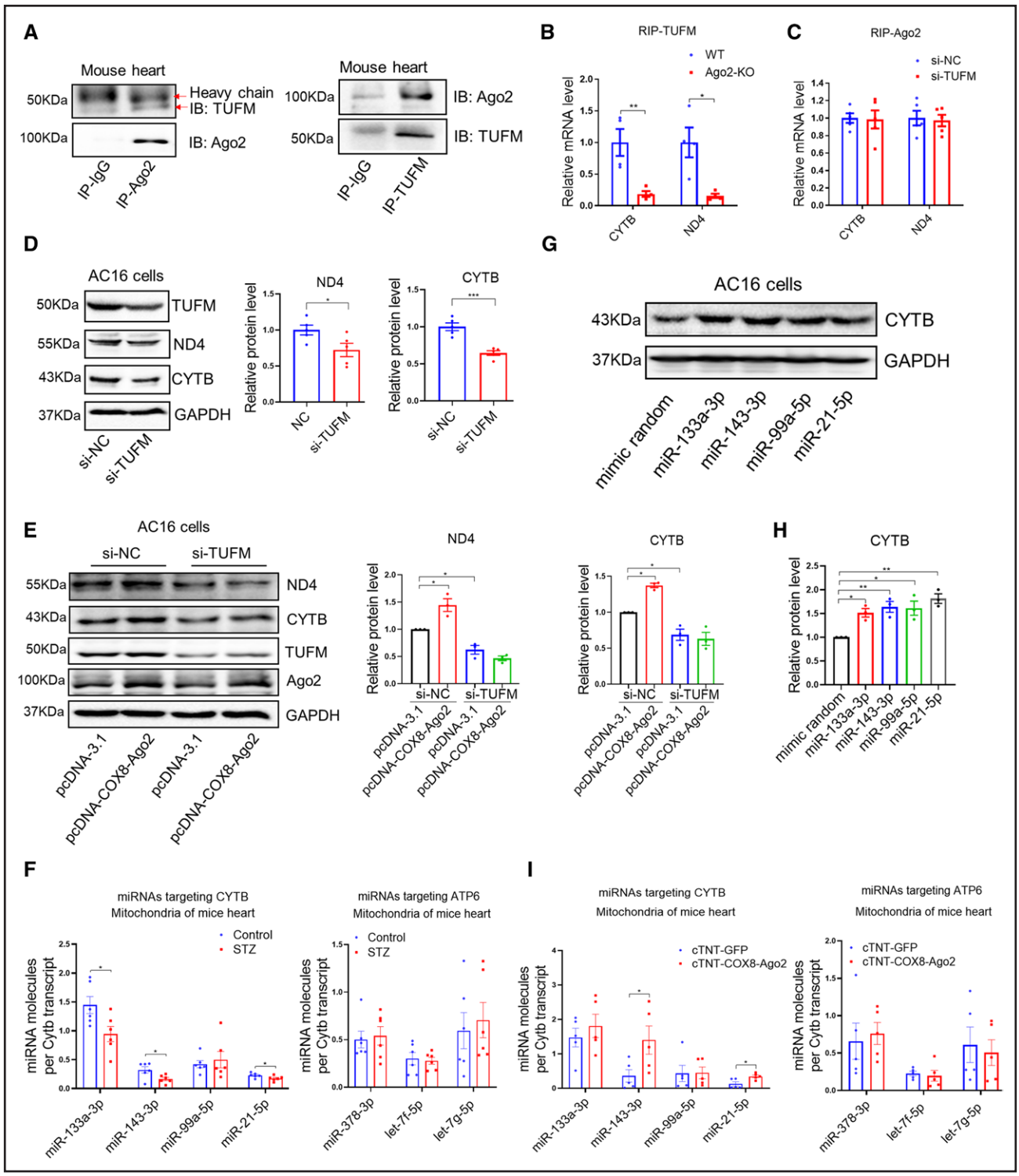

Figure 4. Mitochondrial Ago2 enhanced mitochondrial gene translation by recruiting TUFM with the participation of mitochondrial miRNAs.

A, Western blot analysis of the association between TUFM (Tu translation elongation factor) with Ago2 (Argonaute2) using anti-Ago2 antibody (left) or anti-TUFM (right) in mouse heart. **B**, The association of ND4 (NADH dehydrogenase 4) and CYTB (cytochrome B) mRNAs with TUFM in wild-type (WT) or Ago2 knockout (Ago2-KO) AC16 cells (n=4). **C**, The association of ND4 and CYTB mRNAs with Ago2 under si-NC or si-TUFM conditions (n=5). **D**, Western blot analysis of ND4 and CYTB protein levels in AC16 cells treated with si-TUFM (n=6). **E**, Western blot analysis of ND4 and TUFM protein levels in AC16 cells overexpressing mitochondrial Ago2 under si-NC or si-TUFM conditions (n=3). **F**, Reverse transcription polymerase chain reaction analysis of miRNAs potentially targeting CYTB mRNA (left) and ATP6 mRNA (right) in mitochondria extracted from streptozotocin-treated mouse hearts (n=6). **G** and **H**, Western blot analysis of ND4 and CYTB protein levels in AC16 cells treated with miR-133a-3p, miR-143-3p, miR-99a-5p, or miR-21-5p mimics, respectively (n=3). **I**, Reverse transcription polymerase chain (*Continued*)

Figure 4 Continued. reaction analysis of miRNAs potentially targeting CYTB mRNA (left) and ATP6 mRNA (right) in mitochondria extracted from hearts of streptozotocin-treated mice overexpressing mitochondrial Ago2 (n=5). **B** through **I**, **P*<0.05, ***P*<0.01, ****P*<0.001. **B** through **D**, **F**, and **I**, Student *t* test. **E** and **H**, 1-way ANOVA followed by Tukey multiple comparisons; number of comparisons=6 (for **E**) or 10 (for **H**).

S7). In contrast, miRNAs with TPM <1000 (<0.1% of total miRNAs in mitochondria) were neglected because of their extremely low abundance. The combined relative abundance of 66 miRNAs was ≈96 200 TPM, accounting for 96% of the total mitochondrial miRNAs in mouse heart. We then used RNAhybrid software to search for potential miRNAs that may specifically target the mt-CYTB transcript or other mt-RNAs (Table S4). Of the 66 mitochondrial enriched miRNAs, we identified 4 miRNAs (miR-133a-3p, miR-143-3p, miR-99a-5p, and miR-21-5p) that were potentially targeted to mt-CYTB, 3 potential miRNAs (miR-143-3p, miR-378a-3p, and miR-24-3p) to target ND4, and 3 miRNAs (miR-378a-3p, let-7f-5p, and let-7g-5p) for ATP6 (Table S4). We observed that in mitochondria isolated from type 1 (streptozotocin) diabetic hearts, the total TPM for mt-CYTB–targeted miRNAs showed a decreased trend compared with their relative control (146 766 in control versus 127 989 in streptozotocin), which was validated by quantitative reverse transcription polymerase chain reaction (Figure 4F; Table S5). In sharp contrast, total TPM for ATP6-targeted miRNAs appeared to show an upregulation trend in mitochondria in the streptozotocin diabetic heart (49 226 in streptozotocin versus 51 778 in control), which was further validated by quantitative reverse transcription polymerase chain reaction (Figure 4F; Table S5). Moreover, the downregulation of mt-CYTB targeting miRNAs (miR-143-3p and miR-21-5p) was also observed in mitochondria from db/db heart (Figure S11A; Excel S8). Because mitochondrial miRNAs activated mitochondrial gene expression (Figure 4G and 4H), the specific decrease of mt-CYTB–targeted miRNAs might explain the selective downregulation of mt-CYTB in diabetic heart (Figure 3B). The translational activation effects of these CYTB-targeting miRNAs were independent of GW182 (miR-133a-3p and miR-143-3p in Figure S11B and S11C and miR-21 in our previous article[14]), which is a critical factor for miRNA action in cytoplasm. Moreover, miRNA-mediated CYTB upregulation was lost in Ago2 KO or TUFM knockdown myocytes (Figure S11D and S11E) and mitochondrial Ago2-mediated CYTB upregulation was abolished by miRNA knockdown (Figure S11F). These data suggested that miRNA from the Ago2-miRNA machinery provided the base-pairing information and Ago2 recruited TUFM to promote mt-mRNA translation (Figure S11G). In contrast, the mt-ATP6–targeting miRNAs appeared to be slightly increased in mitochondria from streptozotocin hearts (Figure 4F), which might compensate for Ago2 loss-induced mitochondrial translational repression of ATP6, together resulting in unchanged ATP6 expression in diabetic cardiomyopathy.

Furthermore, upon mitochondrial Ago2 overexpression, the copy number for mt-CYTB–targeted miRNAs increased, whereas mitochondrial miRNAs for ATP6 were unchanged (Figure 4I), which might explain the different regulation effects of mitochondrial Ago2 on mt-CYTB and ATP6 expression. In terms of the regulation of mito-Ago2 on mt-miRNAs, mt-Ago2 appeared to be able to regulate mt-miRNAs levels through a transcriptional manner rather than directly affecting their mitochondrial importing efficiency (see discussion for more details).

Taken together, decreased Ago2 coupled with dysregulated miRNAs might be collectively responsible for remodeling of mitochondrial ETC subunits, among which the specific decrease of mt-CYTB appeared to contribute directly to increased mt-ROS production in diabetic hearts.

Malonylation of Ago2 Impaired Its Mitochondrial Translocation

Having demonstrated the functional relevance and mechanism of mitochondrial Ago2 in regulating its downstream gene mt-CYTB expression and mitochondrial ROS production, we then investigated the upstream mechanism underlying Ago2 mitochondrial translocation. As cytosol Ago2 was unchanged, decreased mitochondrial Ago2 expression appeared to be attributable to decreased mitochondrial importing efficiency. Previous studies have demonstrated that PTM of Ago2 (such as phosphorylation) affected Ago2 cytoplasmic localization and granular distribution in HEK293 cells.[22,23] We therefore explored whether Ago2 PTM is responsible for rearranged Ago2 mitochondrial localization. By Ago2 immunoprecipitation followed by MS analysis of PTM of Ago2, we observed phosphorylation, acetylation, and malonylation modification of Ago2 in mouse hearts (Excel S9; Figure S12A). By Western blotting validation using pan-modification antibodies, we found that Ago2 malonylation was increased in streptozotocin and db/db hearts compared with controls, whereas other modifications remained undetectable or unaltered. Ago2 acetylation was only increased in streptozotocin but not in db/db mice (Figure S12B); Ago2 phosphorylation was detectable with extremely high concentration of antibodies applied but remained unchanged (Figure 5A;Figure S12C and S12D). We performed Western blotting analysis on glycosylation and AGE modification, which were suggested to participate in the progression of diabetic cardiomyopathy,[4,37–41] finding that Ago2 was modified neither by glycosylation nor by AGEs (Figure 5A). In cultured myocytes, Ago2 malonylation was increased by high glucose treatment

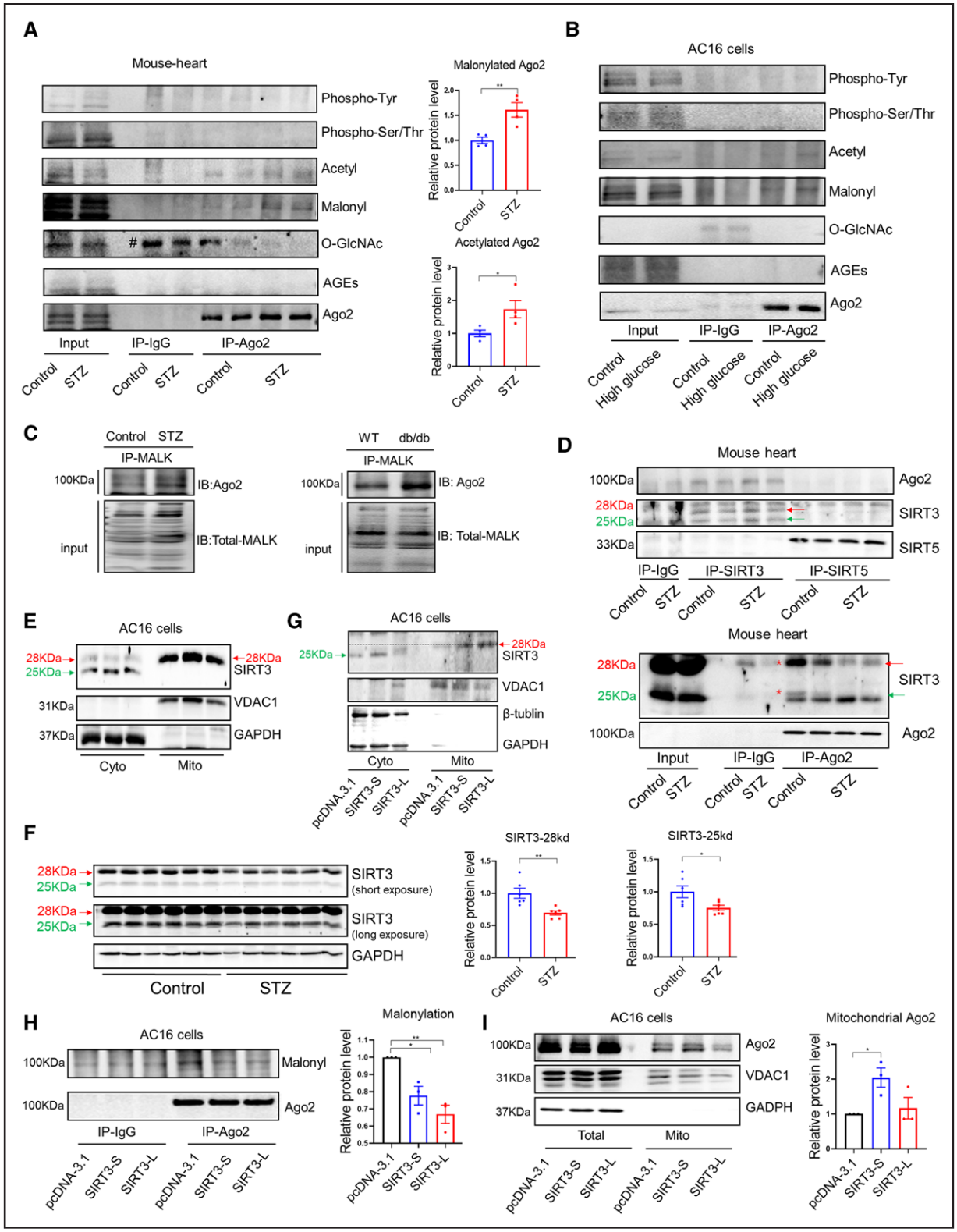

Figure 5. Malonylation of Ago2 impaired its mitochondrial translocation.

A, Immunoprecipitation of cardiac Ago2 (Argonaute2) from streptozotocin (STZ)–treated mice probed with anti-tyrosine phosphorylation (Phospho-Tyr), anti-serine and threonine phosphorylation (Phospho-Ser/Thr), anti-lysine acetylation (Acetyl), anti-lysine malonylation (Malonyl), anti-O-GlcNAcylation (O-GlcNac), and anti–advanced glycation end products (AGEs) antibody, respectively. Quantitative analysis of acetylated and malonylated Ago2 levels are presented in the right panel (n=4). #, nonspecific band. **B**, Immunoprecipitation of Ago2 in high glucose (33.3 mM) treated AC16 cells probed with anti-tyrosine phosphorylation (Phospho-Tyr), anti-serine and threonine phosphorylation (Phospho-Ser/Thr), anti-lysine acetylation (Acetyl), anti-lysine malonylation (Malonyl), anti-O-GlcNAcylation (O-GlcNac), and anti-AGEs, respectively. **C**, Western blot analysis of cardiac malonylated Ago2 levels in streptozotocin-treated mice and db/db mice using anti-lysine malonylation antibody. **D**, (*Continued*)

Figure 5 Continued. Western blot analysis of the binding capacity of Ago2 with SIRT3 (sirtuin 3) or SIRT5 (sirtuin 5) in streptozotocin-treated mouse hearts using anti-SIRT3 and anti-SIRT5 antibody, respectively (**top**). Reverse coimmunoprecipitation using anti-Ago2 antibody is also presented (**bottom**). **E**, Western blot image showing different subcellular localizations of SIRT3 isoforms (28 KDa and 25 KDa in molecular weight) in AC16 cells. **F**, Western blot analysis of cardiac SIRT3 levels in streptozotocin-treated mice. Quantitative analysis of 2 isoforms (28 KDa and 25 KDa) is presented in the right panel (n=6). **G**, Western blot image showing different subcellular localizations of SIRT3 isoforms (28 KDa and 25 KDa in molecular weight) in AC16 cells overexpressing 2 forms of SIRT3 (the longer coding sequence [CDS]: 1002 nt; the shorter CDS: 771 nt), respectively. SIRT3-S: the short form of SIRT3 with a 771 nt-CDS; SIRT3-L: the longer form of SIRT3 with a 1002 nt-CDS. **H**, Western blot analysis of malonylated Ago2 levels using anti-Ago2 antibody in AC16 cells overexpressing 2 forms of SIRT3, respectively (n=3). **I**, Western blot analysis of mitochondrial Ago2 levels in AC16 cells overexpressing 2 forms of SIRT3, respectively (n=3). **A**, **F**, **H**, and **I**, $^{*}P<0.05$, $^{**}P<0.01$. **A** and **F**, Student *t* test. **H** and **I**, 1-way ANOVA followed by Tukey multiple comparisons; number of comparisons=3. Cyto indicates cytoplasm; and Mito, mitochondria.

(Figure 5B). We then performed a reciprocal experiment by using pan-malonylation antibody to capture Ago2, also revealing increased Ago2 malonylation in the diabetic heart (Figure 5C; Figure S12E).

Malonylation is an evolutionarily conserved modification wherein malonyl-CoA is used as a substrate to add a malonyl group to the amino acid lysine.[42,43] We measured malonyl-CoA levels in the diabetic heart. Malonyl-CoA levels were unchanged in type 1 and type 2 diabetic hearts (Figure S12F). Until now, the only reported regulator of malonylation is SIRT5 (sirtuin 5), a member of the histone deacetylase family that can act not only as a demalonylase but also to remove similar acylations, such as succinylation and glutarylation.[44,45] SIRT5 was not associated with Ago2 and did not show dysregulation in diabetic hearts (Figure 5D; Figure S12G and S12H). We then screened for Ago2-associated proteins by using a published HitPredict-database, and 356 proteins were identified to directly interact with Ago2 in HEK293 cells, including SIRT1.[46,47] However, Western blotting analysis showed that SIRT1 was not associated with Ago2 in myocytes (Figure S12I), indicting the demalonylase activity of SIRTs appeared to be cell type–specific. To identify Ago2-associated SIRTs in myocytes, we performed immunoprecipitation analysis to assess the potential association of Ago2 with all SIRTs (SIRT1 through SIRT7) that are reportedly expressed in myocytes,[27] revealing a specific interaction of SIRT3 with Ago2 (Figure 5D; Figure S12J through S12L). We observed 2 distinct bands (~28 KD and ≈25 KD) of SIRT3 by SIRT3 immunoprecipitation (Figure 5D; Figure S13A). We investigated whether different isoforms of SIRT3 were located in different subcellular fractions. We observed that the 28-KD SIRT3 was predominantly expressed in mitochondria, whereas the shorter form of SIRT3 (25 KD) was mainly localized in cytoplasm (Figure 5E; Figure S13B). Both the 28-KD and 25-KD form of SIRT3 protein were significantly decreased in diabetic hearts (Figure 5F; Figure S13C and S13D).

We next investigated whether dysregulated SIRT3 contributed to Ago2 malonylation and mitochondrial translocation. For the gain-of-function studies, we designed and constructed the SIRT3-overexpressing plasmid. SIRT3 appeared to have 5 different alternative splicing transcripts, 2 of which were detectable in the mouse heart by reverse transcription polymerase chain reaction followed by sequencing using 3 primer sets designed to target the 5 transcripts (Figure S13E and S13F). The overexpressing plasmids containing 2 forms of SIRT3 coding sequences (CDS) were constructed (the long CDS of 1002 nt and the short CDS of 771 nt). We found that the cytosol 25 KDa-SIRT3 protein was derived from the short transcript (CDS: 771 nt) whereas the long SIRT3 transcript (CDS: 1002 nt) was processed into the 28-KDa form SIRT3, which was mainly expressed in mitochondria (Figure 5G; Figure S13B). Overexpression of the short SIRT3 led to decreased Ago2 malonylation and increased mitochondrial localization, whereas SIRT3 knockdown showed an opposite effect (Figure 5H and 5I; Figure S13G through S13I). In contrast, long SIRT3 overexpression had no effect on Ago2 mitochondrial localization despite a decreased Ago2 malonylation (Figure 5H and 5I), which might have been attributable to Ago2 malonylation in noncytoplasmic fractions (see the discussion for details). Moreover, short SIRT3-mediated Ago2 demalonylation and mitochondrial translation was not restricted to myocytes, as a similar effect was also observed in HEK293 cells under similar treatment conditions (Figure S14).

Malonylation of Ago2 at Lysine(K)^{440} Led to Detachment of Ago2 From Mitochondrial Transporter Timm17b

SIRT3 is widely accepted as a deacetylase. To rule out the potential effects of acetylation or other forms of Ago2 PTMs on Ago2 mitochondrial translocation, we identified the malonylation site of Ago2 and performed a loss-of-function study. MS analysis coupled with bioinformatics prediction using a published Mal-Lys database revealed 8 potential malonylation sites of Ago2 (1 identified by MS, 7 identified by Mal-Lys database; Table S6), 3 of which were selected for further validation on the basis of the rank of scores. We found that Ago2 point mutation at Lysine(K)^{440} resulted in decreased malonylation of Ago2 (acetylation was not affected) and increased mitochondrial localization (Figure 6A and 6B). Short-form SIRT3 was no longer able to further increase mitochondria translocation of Ago2 that carried the Lysine(K)^{440}

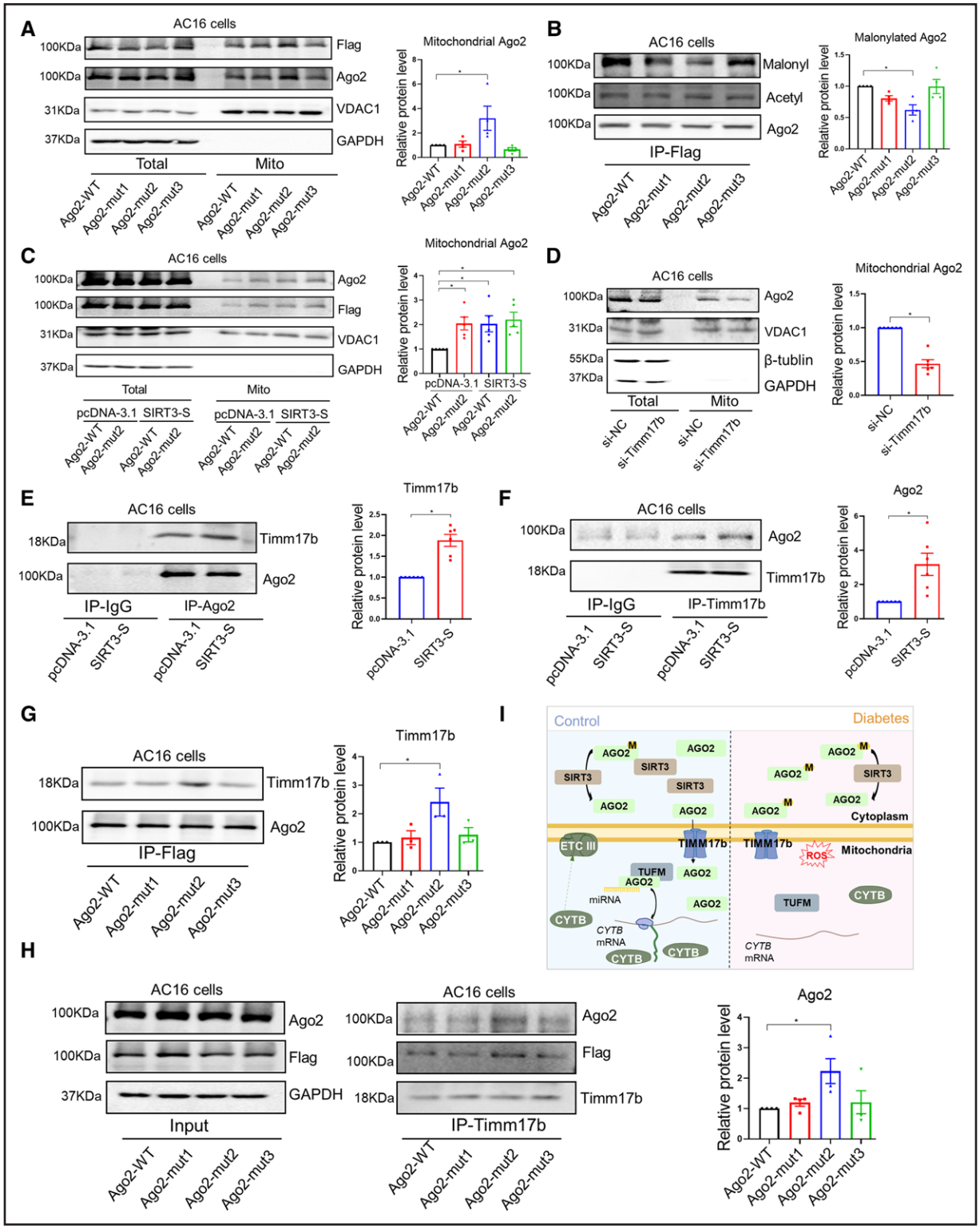

Figure 6. Malonylation of Ago2 at lysine(K)440 led to detachment of Ago2 from mitochondrial transporter Timm17b.
A, Western blot analysis of mitochondrial Ago2 (Argonaute2) levels in AC16 cells transfected with plasmids expressing different mutant forms of Ago2. All forms of Ago2 were fused with flag label (n=4). **B**, Western blot analysis of malonylated Ago2 levels using anti-flag in AC16 cells transfected with plasmids expressing different mutant forms of Ago2 (n=4). **C**, Western blot analysis of mitochondrial Ago2 levels in AC16 cell cotransfected with plasmids expressing Ago2 with a point mutation in Lysine(K)^{440} (Ago2-mut2) and the short form of SIRT3 (sirtuin 3; n=5). **D**, Western blot analysis of mitochondrial Ago2 levels in AC16 cell treated with si-Timm17b (n=6). **E** and **F**, Western blot analysis of Timm17b binding with Ago2 using anti-Ago2 antibody (**E**) in AC16 cells overexpressing the short form of SIRT3 and reciprocal coimmunoprecipitation using anti-Timm17b antibody (**F**; n=6). **G** and **H**, Western blot analysis of Timm17b binding with Ago2 using anti-Flag antibody (**G**) in AC16 cells transfected with plasmids expressing different mutant forms of Flag-tagged Ago2, and reciprocal coimmunoprecipitation using anti-(*Continued*)

Figure 6 Continued. Timm17b antibody (**H**; n=3 or 4). **I**, A model to elucidate the protective role of mitochondrial Ago2 in diabetic cardiomyopathy. In diabetic heart, loss of the short form of SIRT3 in cytoplasm led to increased Ago2 malonylation, impaired Ago2:Timm17b interaction, and compromised mitochondrial Ago2 translocation. Mitochondrial Ago2/miRNAs directly interacted with TUFM (Tu translation elongation factor) to enhance mitochondrial translation. In diabetes, decreased Ago2 in mitochondria hindered CYTB (cytochrome B) translation, which directly contributed to significant increase of mitochondrial reactive oxygen species and cardiac dysfunction. **A** through **H**, *$P<0.05$. **A** through **C**, **G**, and **H**, 1-way ANOVA followed by Tukey multiple comparisons (number of comparisons=6). **D** through **F**, Wilcoxon signed-rank test. Ago2-mut1 indicates Ago2 with a point mutation at Lysine(K)^{425}; Ago2-mut2, Ago2 with a point mutation at Lysine(K)^{440}; Ago2-mut3, Ago2 with a point mutation at Lysine(K)^{468}; and Ago2-WT, wild-type form of Ago2.

point mutation site (Figure 6C). In contrast, point mutation of Ago2 acetylation sites showed no effect on Ago2 mitochondrial translocation (Figure S15), indicating that Ago2 malonylation rather than acetylation mediated its mitochondrial translocation.

We then asked how Ago2 malonylation impaired Ago2 translocation into mitochondria. Ago2 malonylation at Lysine(K)^{440} was located in the linker region (347aa–445aa) of Ago2, which was less characterized compared with the well-studied PAZ and PIWI domains. However, a previous study demonstrated that in HEK293 cells, phosphorylation of Ago2 at serine 387 in the linker region facilitated its localization to processing bodies,[48] indicating that the Ago2 linker region might be responsible for its subcellular localization. Using MS analysis, we identified several integral components of the mitochondrial translocase TIM23 complex, including timm17b, timm29, and timm50 (Excel S6). Timm17b was selected for further validation on the basis of the fold change of immunoprecipitation-Ago2 relative to immunoprecipitation immunoglobulin G. Knockdown of Timm17b led to decreased Ago2 localization in mitochondria (Figure 6D; Figure S16A), indicating that a Timm17b-containing complex might be responsible for importing Ago2 into mitochondria. In cultured myocytes, Ago2 demalonylation by Lysine(K)^{440} point mutation or short SIRT3 overexpression increased the association between Ago2 and Timm17b (Figure 6E through 6H). In contrast, increasing Ago2 malonylation level by SIRT3 knockdown resulted in decreased Ago2-Timm17b interaction, which might further lead to decreased mitochondrial Ago2 translocation (Figure S16B and S16C).

These data reveal that malonylation of Ago2 at Lysine(K)^{440} is responsible for decreased importing of Ago2 into mitochondria by the Timm17b-tontaining transporter.

DISCUSSION

We uncovered the critical roles of mitochondrial Ago2 in diabetes-triggered cardiac dysfunction. We found that the SIRT3-Ago2-CYTB axis links glucotoxicity to ETC imbalance in the heart. These results provide new mechanistic insights and suggest a potential strategy to develop mitochondria-targeting therapy to treat diabetes-associated cardiovascular complications (Figure 6I).

Mitochondrial Imbalance in Diabetic Cardiomyopathy

Mitochondrial dysfunction in the diabetic heart has been extensively studied. Impaired mitochondrial function was initially reported when Kuo et al[49] showed depressed state 3 respiration in db/db heart mitochondria. Decreased mitochondrial respiration and reduced protein expression of the ETC components reduce ATP and increase mt-ROS production in type 1 and type 2 diabetic mice.[15,50–53] Additional mitochondrial defects, such as impairment of mitochondrial Ca^{2+} handling, opening of mitochondrial permeability transition pore, and mitochondrial autophagy in diabetes, also may play critical roles in the development of cardiac dysfunction.[54–57] However, conflicting data show mitochondrial biogenesis signaling as being amplified or decreased,[58,59] which might reflect different diabetic models or different stages of cardiac dysfunction. The key question is which targets/genes harbor the highest potential for clinical application. In the cardiovascular system, complex I and complex III of the ETC are the major sites for ROS production, and multiple studies suggest that complex III is more important than complex I in generating mt-ROS in the heart.[14,33] Complex III consists of 11 subunits encoded by nuclear and mitochondrial DNA. Only 3 of these subunits (CYTB, CYC1, and UQCRFS1) contain redox prosthetic groups that may be directly involved in the electron transport contributing to ROS production.[60,61] In diabetes, CYTB, the only mtDNA-encoded subunit of complex III, was decreased more dramatically than CYC1 and UQCRFS1 in mitochondria isolated from diabetic heart. Therefore, decreased mt-CYTB in the diabetic heart appears to be the "shortest piece of wood" of a "buckets effect" in the control of ROS production. Restoring CYTB expression in the diabetic heart might be a therapeutic strategy for treating cardiac dysfunction in diabetes. However, overrestored CYTB might be as detrimental as CYTB insufficiency in the heart, because a recent study revealed that mitochondrial imbalance appeared to be the cause, rather than the altered function of specific genes involved, as evidenced by data showing that upregulation or downregulation of the same ETC subunits similarly augment ROS production.[13] Therefore, restoring mitochondrial ETC subunits expression requires careful investigation of the dose-dependent effects to avoid ETC overexpression-induced mitochondrial imbalance.

Ago2/miRNAs Action in Mitochondria

Imbalanced changes of mitochondrial ETC subunits in the diabetic heart have been reported before. Vazquez et al[62] demonstrated decrease of NDUFB8 (a subunit of complex I), and unchanged level of SDHB (a subunit of complex II), UQCRC2 (a subunit of complex III), and ATPase subunit A (a subunit of complex IV) in streptozotocin-induced type 1 diabetic hearts. Our previous study also revealed uncoordinated downregulation of mitochondrial gene-encoded subunits in db/db type 2 diabetic hearts.[15] However, the mechanisms underlying mitochondrial imbalance in diabetes remained unclear. In the current study, we showed that dysregulated Ago2 coupled with rearranged mitochondria localized miRNAs together contributed to imbalanced mitochondrial ETC subunits expression. Mitochondria-localized miRNAs were able to directly enhance mitochondrial translation, and 3 critical requirements were revealed for converting miRNA-dependent translational repression to activation: lack of the cap at the 5′ end, lack of a typical poly(A) tail at the end, and detachment of GW182 from an Ago protein.[20] Mitochondrial transcripts fulfill all these requirements. We found that mitochondrial Ago2/miRNAs directly interacted with translation elongation factor TUFM to enhance mitochondrial translation. miRNAs exerted their function through a base-pairing rule with high sequence specificity, which might explain imbalanced ETC subunit expression during disease. In the diabetic heart, the dysregulations of miRNAs in mitochondria were consistent with the changes in the whole heart (data not shown), indicating that cytoplasmic miRNAs are able to translocate freely into mitochondria. Mitochondrial Ago2 overexpression resulted in the rearrangement of a cluster of miRNAs in mitochondria, which were also consistent with their pre-miRNA expression (data not shown), indicating that mitochondrial Ago2 appears to regulate miRNA levels through a transcriptional manner rather than by directly affecting their mitochondrial importing efficiency. It is possible that mitochondrial Ago2 interacts with or sequesters some transcriptional factors for miRNAs, leading to subcellular relocalization of these transcriptional factors to prevent their action in the nucleus to regulate miRNA production. We have preliminary data to show mitochondrial Ago2 interacted with YY1 to prevent YY1 translocation into nucleus. The loss of YY1 in nucleus led to increased transcription of miR-21. Another possible mechanism may be that mitochondrial Ago2 increases ATP production (and possibly other metabolites such as acetyl-CoA), leading to altered YY1 phosphorylation (or acetylation) status and rearranged YY1 (or other transcription factors) activity. Overall, then, the detailed mechanisms underlying mitochondrial Ago2-mediated regulation on a large scale of miRNAs remain to be determined, representing new fields for further research.

Downregulation of Ago2 in Mitochondria

In the early stage of diabetes, the mRNA levels and Ago2 mRNA and cytoplasmic form of Ago2 protein were not changed in either type 1 or type 2 diabetic mice; therefore, the decreased localization of Ago2 in mitochondria appears to be attributable to impaired translocation from cytoplasm into mitochondria. However, in the late stage of diabetic cardiomyopathy (>6 months), transcriptional dysregulation of Ago2 was observed and Ago2 mRNA was decreased in the type 1 diabetic heart; but, in contrast, was increased in the type 2 diabetic heart. Therefore, glucose, insulin, and lipids that are distinctly dysregulated in type 1 and type 2 diabetic hearts appear to exert complicated (or even conflicting) effects on Ago2 expression. Despite the distinct expressional patterns of total Ago2 between type 1 and type 2 diabetic hearts, the decrease of Ago2 in mitochondria was consistent and persistent between the 2 diabetic models. Hyperglycemia alone was already able to impair Ago2 translocation into mitochondria. Moreover, this study revealed that restoring mitochondrial Ago2 level protected cardiac function in both type 1 and type 2 diabetic models, indicating that targeting mitochondrial Ago2 is beneficial for preventing diabetes-induced cardiac dysfunction under insulin insufficiency and insulin resistance. However, it would be beneficial to design further studies to identify the effects of glucose, insulin, and lipids (alone or in combination) on Ago2 transcription and translocation.

Short Form of SIRT3 Mediates Ago2 Demalonylation in Cytoplasm

Previous studies have demonstrated that PTMs of Ago2 (such as phosphorylation) affect Ago2 cytoplasmic localization and granular distribution in HEK293 cells.[22,23] However, instead of phosphorylation (with extremely high concentration of antibodies applied, cardiac Ago2 phosphorylation signal was slightly visible, but remained unchanged in diabetes), we found that in the heart, Ago2 translocation was mediated by Ago2 malonylation, indicating that PTMs of Ago2 are highly cell type–specific. Protein malonylation can be functional, as exemplified by a previous study showing that malonylation of glycolytic enzyme GAPDH in marrow-derived macrophages has an effect on proinflammatory cytokine production by modulating both its enzymatic activity and RNA-binding capacity.[63] Malonylation has also been linked to biologic processes such as fatty acid synthesis, mitochondrial respiration, and angiogenesis.[64–66] An established regulator for malonylation is SIRT5, which catalytically removes malonyl groups from the lysine side chain of protein substrates in mouse liver.[45,67] However, in myocytes, SIRT3, rather than SIRT5, was responsible for removing malonyl groups from Ago2, again indicating that malonylation on specific proteins is highly tissue- and cell type–

dependent. In terms of SIRT3, the mitochondrial targeting sequence–containing full-length 44 KDa-SIRT3 (long SIRT3) is proteolytically processed in the mitochondrial matrix to a 28-KDa activated product. This processing can be reconstituted in vitro with recombinant mitochondrial matrix processing peptidase and is inhibited by mutation of arginines 99 and 100. The unprocessed form of full-length SIRT3 is enzymatically inactive.[68] In mitochondria, SIRT3-mediated deacetylation-activated ETC complexes, including NDUFA9 (complex I) and SDHA (complex II), are cardioprotective, as illustrated by studies showing that SIRT3 deficiency aggravates cardiac dysfunction in diabetes and in a pressure overload model.[69–71] A short form of SIRT3 (short-SIRT3) lacking the N-terminal mitochondrial localization signal also has deacetylase activity,[72] although it appears to be less potent in protecting the heart from doxorubicin-induced dilated cardiomyopathy compared with long SIRT3.[73] The molecular weight of short SIRT3 was predicted to be 28 KDa, similar to cleaved (activated) long SIRT3. However, we observed an extra smaller band (≈25 KDa) with short-SIRT3 overexpression, indicating that short SIRT3 is further processed despite the lack of the well-known matrix processing peptidase cleavage site. Supporting this hypothesis, a previous study also observed 2 distinct protein bands after short-SIRT3 overexpression[74]; another study showed that transfection of the vector containing the mutated SIRT3 matrix processing peptidase cleavage site into mammalian cell led to the appearance of novel cleavage products, suggesting additional processing events.[68] The new cleavage sites and processing peptidase for short SIRT3 remain to be revealed.

The distinct expression patten of the short SIRT3 between tissues might explain why global malonylated protein levels were unchanged in SIRT3 KO lenses compared with wild-type lenses.[75] We noticed that the short form of SIRT3 (~25 KDa) was undetectable in lenses, indicating that the expression of the short SIRT3 varied dramatically among tissues; therefore, it would be beneficial to perform cellular and subcellular studies for SIRTs (including SIRT3) to reveal the detailed mechanisms underlying highly selective PTMs of targeted protein in different cells. Both the short and long forms of SIRT3 had decreased Ago2 malonylation levels, whereas only the short SIRT3 increased Ago2 mitochondria translocation. To explain this, we noticed that long SIRT3 was predominantly expressed in mitochondria and the nucleus, but not cytoplasm.[74] Therefore, decreased Ago2 malonylation mediated by long SIRT3 overexpression might take place in noncytoplasmic fractions such as the nucleus. Whether nuclear malonylation of Ago2 affects its RNA binding or DNA binding property represents a new research area.

As characterized in SIRT5, the preference for succinyl and malonyl groups was explained by the presence of Tyr102 and Arg105 in the active site, to the extent that presumably other sirtuins with the conserved Arg and Tyr should have NAD (nicotinamide adenine dinucleotide)–dependent desuccinylase or demalonylase activity.[45] A similar presence of Tyr(69) and Arg(72) was observed in the short-SIRT3 active region, although their function remains to be elucidated. However, SIRT7, which was reported to have desuccinylase activity that functionally links to chromatin compaction,[76] carried no such Tyr and Arg in the active site. Therefore, other mechanisms might also be involved in SIRT-mediated desuccinylation or demalonylation; this requires further investigation.

As to the mechanisms underlying decreased SIRT3 expression in diabetes, we noticed that previous studies have demonstrated that AGEs were able to decrease SIRT3 expression.[77] Because AGEs are formed by irreversible nonenzymatic reactions, the persistence of accumulated AGEs reportedly participates in "hyperglycemic memory," a clinical phenomenon in which patients with diabetes are prone to develop persistent diabetic complications despite controlled blood glucose levels.[78] We did observe persistent decrease of SIRT3 in streptozotocin heart even after glucose normalization with insulin (Figure S16D). As such, detailed mechanisms underlying AGEs (or other molecules)–mediated SIRT3 downregulation (whether transcriptional or posttranscriptional), and the potential roles of SIRT3-mito-Ago2/miRNA-CYTB axis in "hyperglycemic memory," are intriguing subjects for future research.

Therapeutic Potential of the SIRT3-mito-Ago2/miRNA-CYTB Axis

Treatment strategies against diabetes-induced cardiac dysfunction might include SIRT3 activation, mitochondrial Ago2 overexpression, mitochondrial miRNA overexpression, and CYTB overexpression. The sirtuin family of nicotinamide adenine dinucleotide (NAD+)–dependent deacylases (SIRT1–SIRT7) is thought to be cardioprotective, and studies have shown that activation of sirtuins or NAD+ repletion induces angiogenesis, insulin sensitivity, and other health benefits in a wide range of age-related cardiovascular and metabolic disease models.[79] However, in terms of SIRT3, there is a lack of specific sirtuin activating compounds, because most sirtuin activating compounds for SIRT3 are pan–sirtuin activating compounds that activate SIRT1 more potently.[80] This raises concerns because SIRT1 has apparently opposing roles in the heart; low to moderate overexpression protects against age-related hypertrophy, fibrosis, and cardiac dysfunction, whereas a high level of overexpression results in cardiomyopathy and hypertrophy.[81,82] Similar to SIRT1, SIRT3 is also a pan-deacetylate that functions in multiple cellular fractions, such as mitochondria, the nucleus, and cytoplasm, to regulate expression of large amounts of genes. Therefore, it is possible that SIRT3 activation might also have opposing roles in the heart, which requires further investigation to uncover the

dose-dependent or localization-dependent roles of SIRT3 and other SIRTs. Another treatment option is to directly overexpress the downstream target, mt-CYTB. However, the codons in mitochondria are markedly different from those in cytoplasm, and it is unclear whether exogenous vectors can successfully overexpress CYTB in mitochondria, and even if so, whether these exogenous CYTB can be assembled efficiently into the ETC complex. Another strategy is to target mitochondrial miRNA for disease intervention; however, global overexpression of a specific miRNA might lead to unexpected off-target effects in other cells or other subcellular fractions because of the lack of established mitochondrial localization signals in miRNAs. The subcellular localization signals for proteins are well-characterized and Ago2 specifically overexpressed in the mitochondria appears to protect the heart through preserving CYTB and mitochondrial homeostasis in diabetes. Meanwhile, the use of rAAVs as delivery vectors is safe,[83] as exemplified by the US Food and Drug Administration approval of Luxturna and Zolgensma. Therefore, delivery of exogenous Ago2 with a mitochondrial signal peptide by rAAV9 vector appeared to be a promising therapeutic strategy against diabetes-induced cardiac dysfunction.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Methods
Tables S1–S10
Figures S1–S16
Excel Files S1–S9
References 84–97

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