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Natural quinones regulate mitochondrial DNA organization which helps resist mitochondrial dysfunction

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Abstract

Mitochondria contain their own DNA (mtDNA) which is essential for respiratory function. Several copies of mtDNA and the DNA binding proteins form structures called nucleoids, however, the molecular details and the pathophysiological significance of nucleoids remain ambiguous. Here, I identified a mitochondrial disease-causing gene ADCK4/COQ8B, essential for ubiquinone biosynthesis, to be involved in nucleoid organisation and maintenance of respiratory chain complexes. Other naturally occurring quinones without electron carrying ability in the mammalian electron transport system, like menaquinone-4 and phylloquinone, can also support nucleoid formation, suggesting this novel role of quinones is independent of their traditional functions. Quinones mediate mtDNA packaging on inner membrane with mitochondria-specific phospholipid cardiolipin. My *in vivo* data indicates that this quinone-based nucleoid maintenance protects against cardiotoxicity caused by the anti-cancer drug doxorubicin, without affecting its antiproliferative potency. These findings present a novel approach of mitochondrially targeted therapy via regulating nucleoid morphology, not only for mitochondrial diseases but also in more general pathological conditions like heart failure.

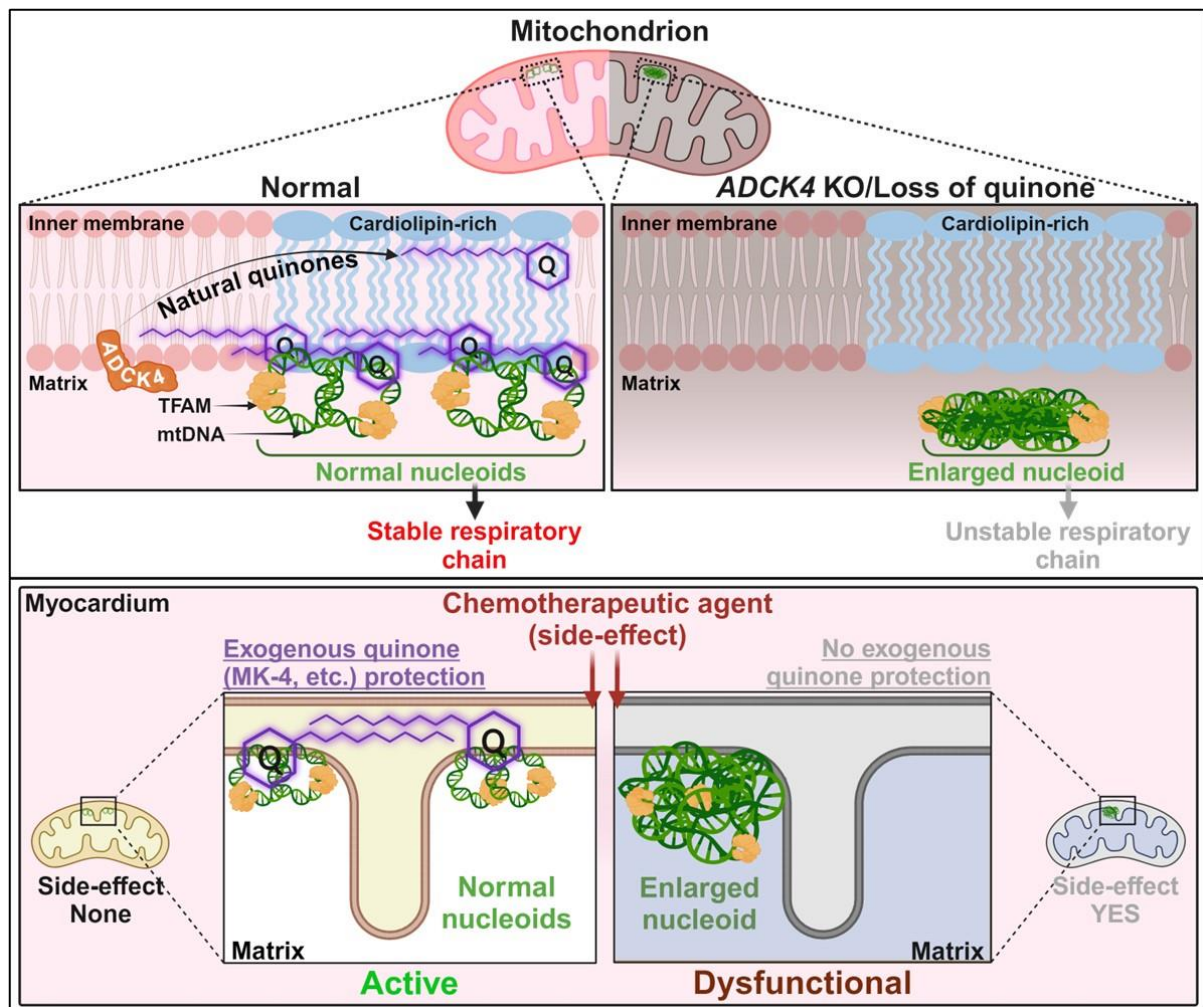
Keywords

Mitochondrial DNA; Nucleoid; Natural quinone; Mitochondrial disease; Respiration; Doxorubicin

Highlights

- ADCK4 (also known as COQ8B), a mitochondrial disease causing gene, is crucial for maintaining nucleoid structure.
- Biological quinones regulate nucleoid morphology irrespective of their traditional functions.
- Cardiolipin and ubiquinone mediate packaging of mtDNA nucleoids on mitochondrial inner membrane.
- Doxorubicin-induced cardiotoxicity is resisted by natural quinones via maintaining proper nucleoid structure.

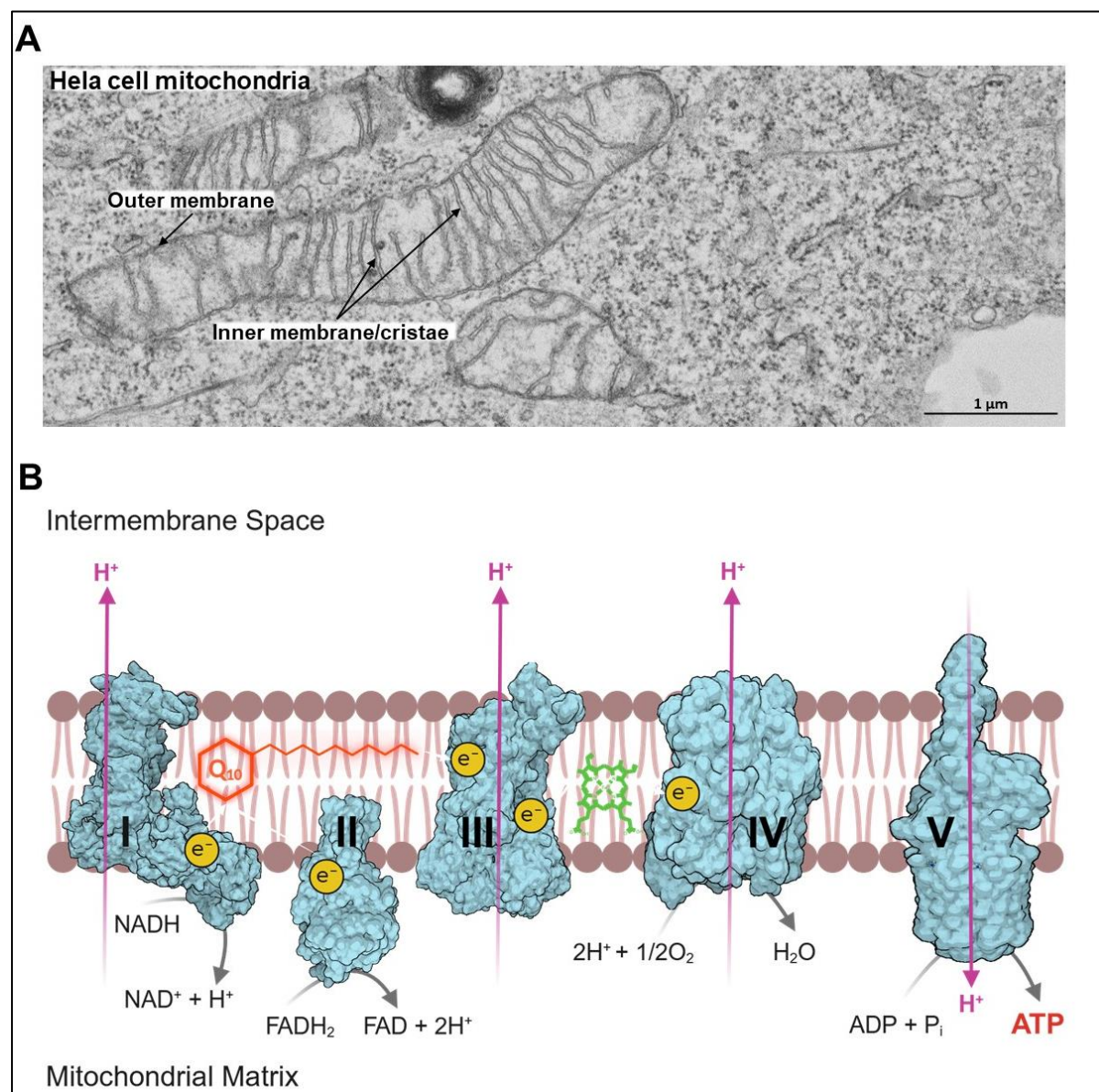
Synopsis



Although mitochondrial DNAs are complexed into nucleoid structures, questions remain regarding the molecular details of its biogenesis and significance. This thesis uncovers a non-canonical role of natural quinones in nucleoid morphology and function.

Introduction

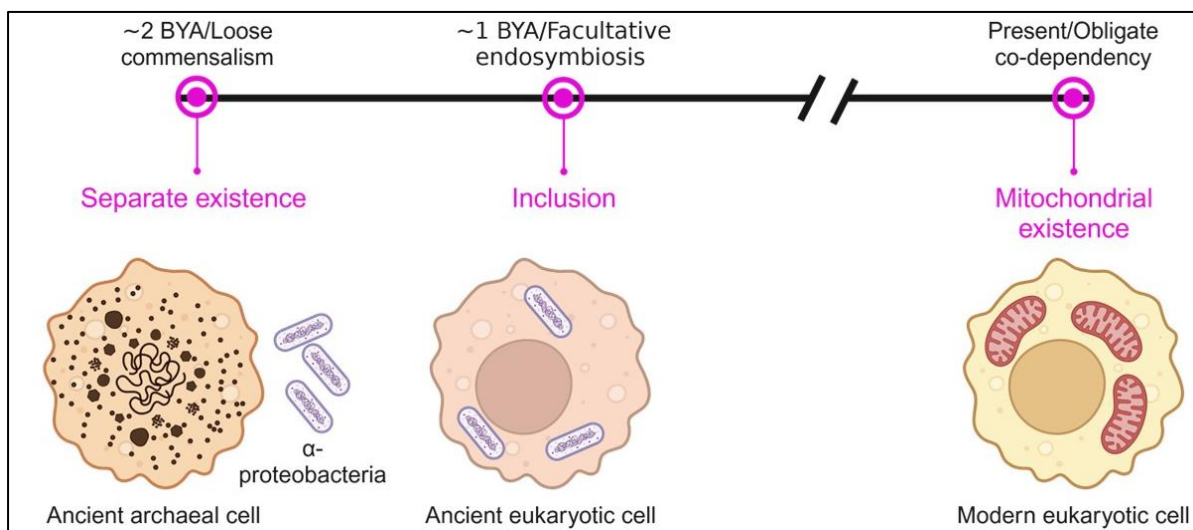
Mitochondria are double membrane bound organelles (Introductory Fig 1A) inside eukaryotic cells that produce ATP, by oxidative phosphorylation, which is essential for various cellular functions. This process starts with the utilization of the products of glucose catabolism or glycolysis, which happens in the cytosol, followed by their transfer into the mitochondria where they get involved in the tricarboxylic acid (TCA) cycle. The final products of TCA cycle, namely nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂). These molecules, in turn act as electron sources for the respiratory chain complexes within the electron transport chain (ETC) inside the mitochondrial inner membrane (Introductory Fig 1B). The flow of electrons across this electron transport chain results in the eventual synthesis of ATP¹.



Introductory Figure 1. Powerhouse of the cell and its origins. A) Electron microscopy image of mammalian mitochondria. They are bound by an outer and an inner membrane, where the latter is

further folded into distinct invaginations called cristae. B) Pictorial representation of the Electron Transport Chain in mammals. Five Respiratory (Electron Transport Chain) Complexes exist in total from I to V which result in ATP production via oxidative phosphorylation. Complexes I to IV are responsible for maintaining a high proton (H^+) concentration in the mitochondrial intermembrane space against the concentration gradient of protons whereas Complex V uses the built-up proton gradient to produce ATP via using ADP and inorganic phosphate (P_i). The maintenance of the proton gradient in the mitochondrial intermembrane space is coupled with the reactions that result in the flow of electrons amidst the Complexes I to IV. Ubiquinone is responsible for carrying the electrons from the iron-sulphur clusters of Complexes I and II (which causes the reduction of ubiquinone to ubiquinol) and transfer it to Complex III (which results the oxidation of ubiquinol back to ubiquinone). Cytochrome c then transfers the electrons from Complex III to Complex IV where it causes the uptake of oxygen and its subsequent reduction into water which is released as a by-product.

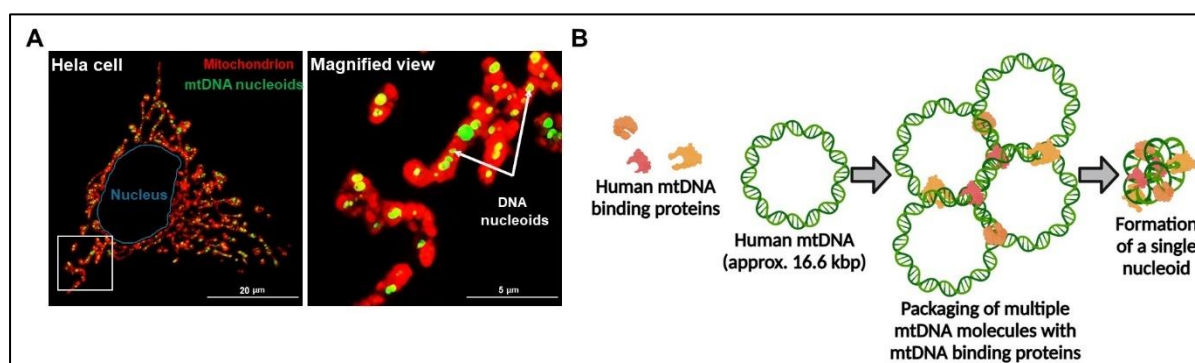
Not only that, mitochondria are considered as hotspots for cellular metabolism and also play important roles in calcium homeostasis², apoptosis³, aging⁴ and immune responses⁵, thus acting as multi-functional organelles. This is the output of billions of years of obligate coexistence with ancient cells which resulted in a symbiotic relationship (Introductory Fig 2). Multiple hypotheses have been put forward regarding the origin of mitochondria. Some believe that the ancestor of mitochondria invaded ancient archaeans⁶, whereas others hypothesize that it was rather an engulfment done by ancient eukaryotic cells⁷. But what is certain is that the ancestors of mitochondria were α -proteobacteria⁸.



Introductory Figure 2. Schematic suggestion of the evolution of mitochondria from α -proteobacteria. They used to exist independently until one invasion event when they entered the ancient archaeal cell and started having symbiotic relationship. Billions of years of co-existence and mutual evolution to adapt to each other finally led to the rise of the modern eukaryotic cell which contains mitochondria in the form of mature organelles which are both dependent on each other for survival.

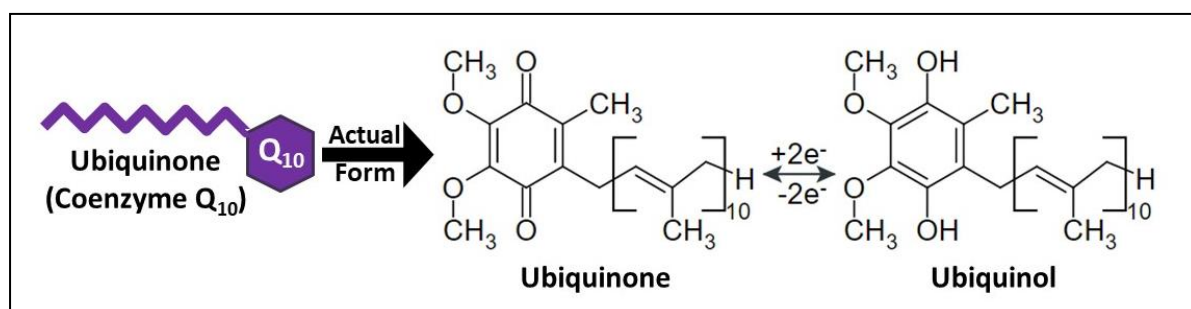
Mitochondria was first identified in the middle of the 19th century⁹ and further investigations, not surprisingly, revealed the existence of mitochondrial DNA (mtDNA) as well¹⁰. Human mtDNA is a circular DNA consisting of about 16.6 kilobase-pairs, and has its own replication, transcription and translation machineries, inside the mitochondrial matrix¹¹. Despite being small, it encodes 13 protein subunits of the respiratory chain complexes I, III, IV and ATP synthase. It also encodes 2 ribosomal

nucleoids with reduced oxidative phosphorylation for ATP synthesis^{21,22}. Other factors like ATAD3 have also been reported to have mtDNA binding capacity and thus control nucleoid organization^{23,24}. Although nucleoids have been linked to mtDNA transcription²⁵ and replication²⁶, molecular details of its biogenesis and structure formation are still less understood. Furthermore, the significance of nucleoids to mark mitochondrial fission sites has also been reported²⁷. Previous reports showed that mitochondrial fission deficiency causes clustering of nucleoids which leads to abnormal mtDNA distribution, resulting in abnormal respiratory complex formation²⁸, cell death²⁹, and impaired murine neonatal heart development³⁰. However, the significance behind the dynamic nature of nucleoids regarding its distribution or structural maintenance inside the living body has not been clarified yet. Apart from a singular previous report³¹, the relationship between nucleoid structure/distribution and pathogenicity remains poorly understood since the dynamic nature of nucleoids in pathological conditions has been long overlooked.



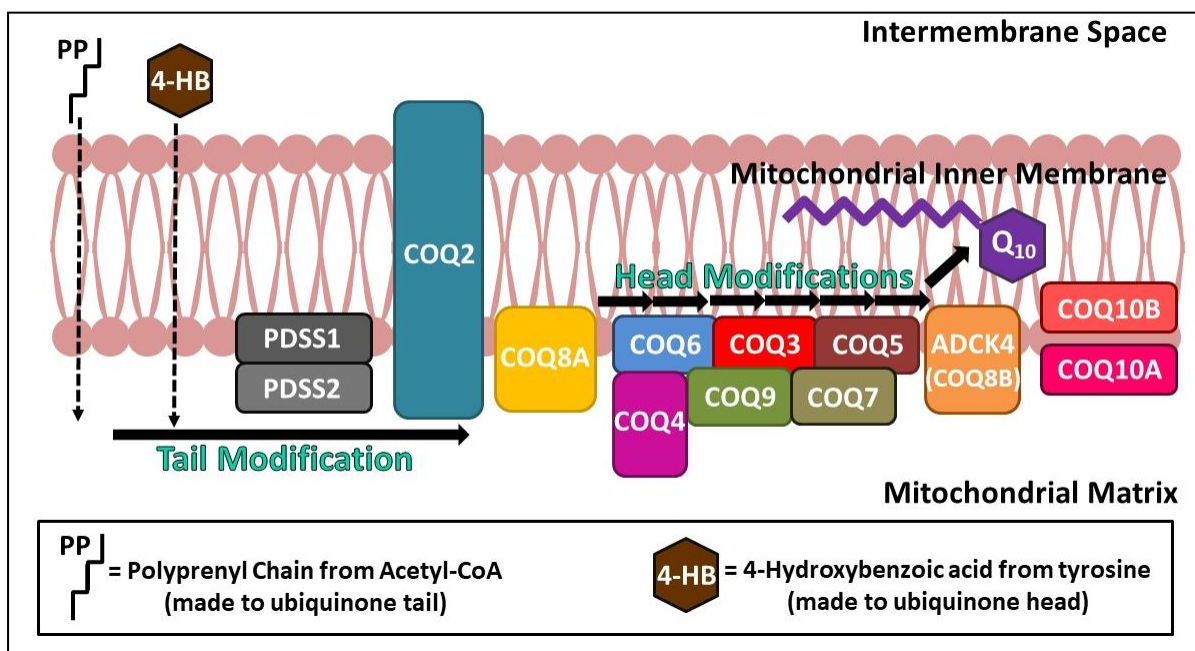
Introductory Figure 4. The appearance of human mtDNA as nucleoids in mammalian cells. A) Fluorescence microscopy image of a single living HeLa cell which shows how the mitochondria (in red) and mitochondrial nucleoids (in green) appear inside a mammalian cell. B) Graphical representation of the formation of a single nucleoid. Multiple copies of the mtDNA are complexed and packaged with various mtDNA binding proteins which results in the formation of the compact nucleoid structures.

Ubiquinone/coenzyme Q₁₀ (Q₁₀) is an essential metabolite for electron transport chain and was discovered first from lipid extracts of beef heart mitochondria³². Now, it is well known that quinones are widely found in bacterial membrane and mitochondrial inner membrane³³. Q₁₀ is a major cyclohexadienedione in humans (Introductory Fig 5); its chemical structure contains one quinone group and ten isoprenyl units³⁴.



Introductory Figure 5. Schematic representation of the structure of ubiquinone. Ubiquinone exists reversibly as ubiquinol which is formed as a result of redox reactions.

The biosynthesis of ubiquinone involves three major steps – the synthesis of the ring structure from the essential amino acid tyrosine that later forms the hydrophilic head, formation of the isoprenoid side chain from acetyl-CoA residues via the mevalonate pathway that later forms the hydrophobic tail, and finally condensation of and modification these structures³⁵. The head and tail regions are imported to the mitochondrial inner membrane and matrix where they are modified and put together by the Coenzyme Q₁₀ biosynthesis complex (Introductory Figure 6). Traditionally, Q₁₀ has two functions. One is that it acts as a mobile electron carrier through the mitochondrial electron transport chain in inner membrane. After accepting electrons from respiratory complexes I and II, ubiquinone is converted to its reduced form, ubiquinol. Ubiquinol then transports the electrons to complex III which, relays the electron flow downstream and after some steps, results in ATP production via oxidative phosphorylation³⁶. The other function is that of a lipid permeable antioxidant; a function it fulfils by quenching the reactive radical species formed as byproducts of mitochondrial lipid metabolism. Thus, Q₁₀ is used popularly as various supplements, as well as a therapeutic agent for mitochondrial diseases³⁷.



Introductory Figure 6. Pictorial representation of the Coenzyme Q₁₀ (ubiquinone/ Q₁₀) Biosynthesis pathway in mammals. The decaprenyl (polyprenyl) chain and the benzenoid obtained from Acetyl-CoA and tyrosine/phenylalanine metabolism is imported into the mitochondria from the cytosol. At the initial stages, the decaprenyl chain is attached (by PDSS1, PDSS2 and COQ2) to the benzenoid compound of 4-hydroxybenzoic acid which ultimately forms the hydrophobic tail and hydrophilic head regions of ubiquinone. The head region of the resulting compound is then modified by the core subunits of the Coenzyme Q₁₀ Biosynthesis complex by subjecting it to a series of methylation (by COQ3 and COQ5) and hydroxylation (by COQ6 and COQ7) reactions. The final product formed is ubiquinone which is released into the mitochondrial inner membrane is probably carried to specific locations for its proper functionality (probably by COQ10A and COQ10B).

Here I elaborated an additional function of ubiquinone and other structurally similar natural quinones like menaquinone-4, in the form of stabilising the morphological state of nucleoids on the inner membrane, which thereby leads to the maintenance of the

electron transport chain activity. My study also shows that the quinone-based maintenance of nucleoids also has significance of protecting against cardiac failure, thus, has enough potential to be translated into a novel approach of mitochondrially targeted therapy via regulating the morphology and dynamics of nucleoids using natural quinones. Since these molecules are easily available, they can be put to therapeutic use with immediate effect, curtailing the time needed as in cases of novel drug development.

Results & Figures

RNAi screening of mitochondrial disease-causing genes revealed ADCK4 as a regulator of nucleoid morphology

Until now, the pathophysiological role of nucleoid morphology was not clarified, and it was also unclear whether nucleoid enlargement could cause pathogenesis directly. Therefore, in order to identify mitochondrial diseases-causing genes which were related to the nucleoid morphology, I performed an RNAi screening. As Kohda and colleagues reported in 2016, exome analyses using high-throughput sequencing in patients with childhood-onset mitochondrial respiratory chain complex deficiencies revealed a set of 99 candidate genes of nuclear genome-encoded mitochondrial diseases-causative genes¹⁷. I utilised three independent siRNAs for each gene, and after transfecting HeLa cells with the respective siRNAs, I performed live cell fluorescence microscopy imaging of nucleoids stained by SYBR Green I (Fig 2A) and identified 14 genes that significantly affected nucleoid morphology (Fig 2B and Fig 1A,B).

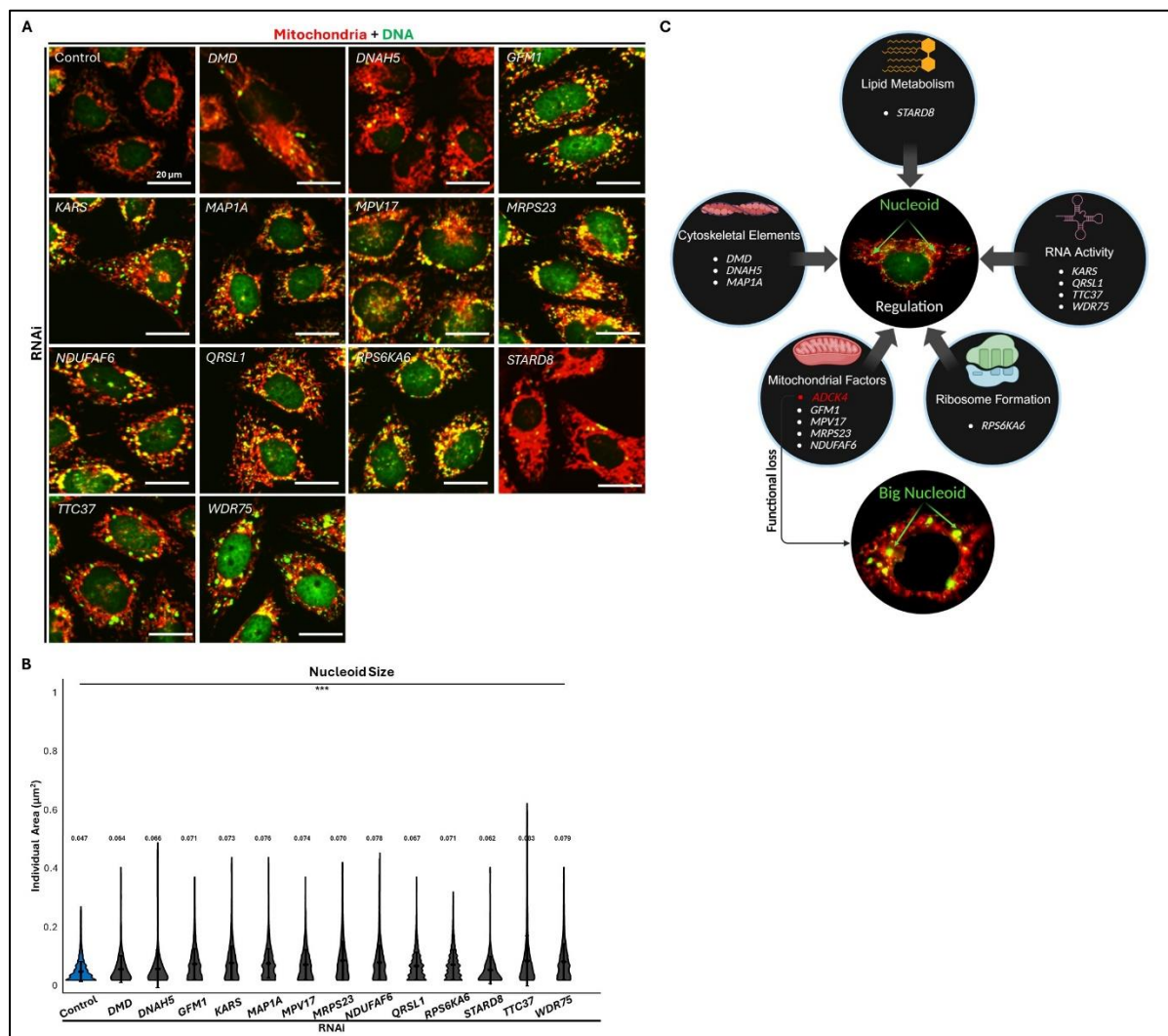


Fig 1. List of mitochondrial nucleoid regulators other than *ADCK4* obtained from RNAi screening on HeLa cells. A) Fluorescence microscopy images (mitochondria, red; nucleoids, green) under 20x

magnification (scale bar 20 μm) and graph showing quantification of B) nucleoid sizes (n =more than 50 cells) of experimental RNAi cells. C) Graphical representation of the factors from different pathways in nucleoid regulation irrespective of mitochondrial localization. Error bars are shown in standard deviation values. All comparisons with control set are statistically significant at $***P<0.001$; where P values were determined by Kruskal-Wallis and Dunn's tests. Decimal numbers indicate the mean values of different populations.

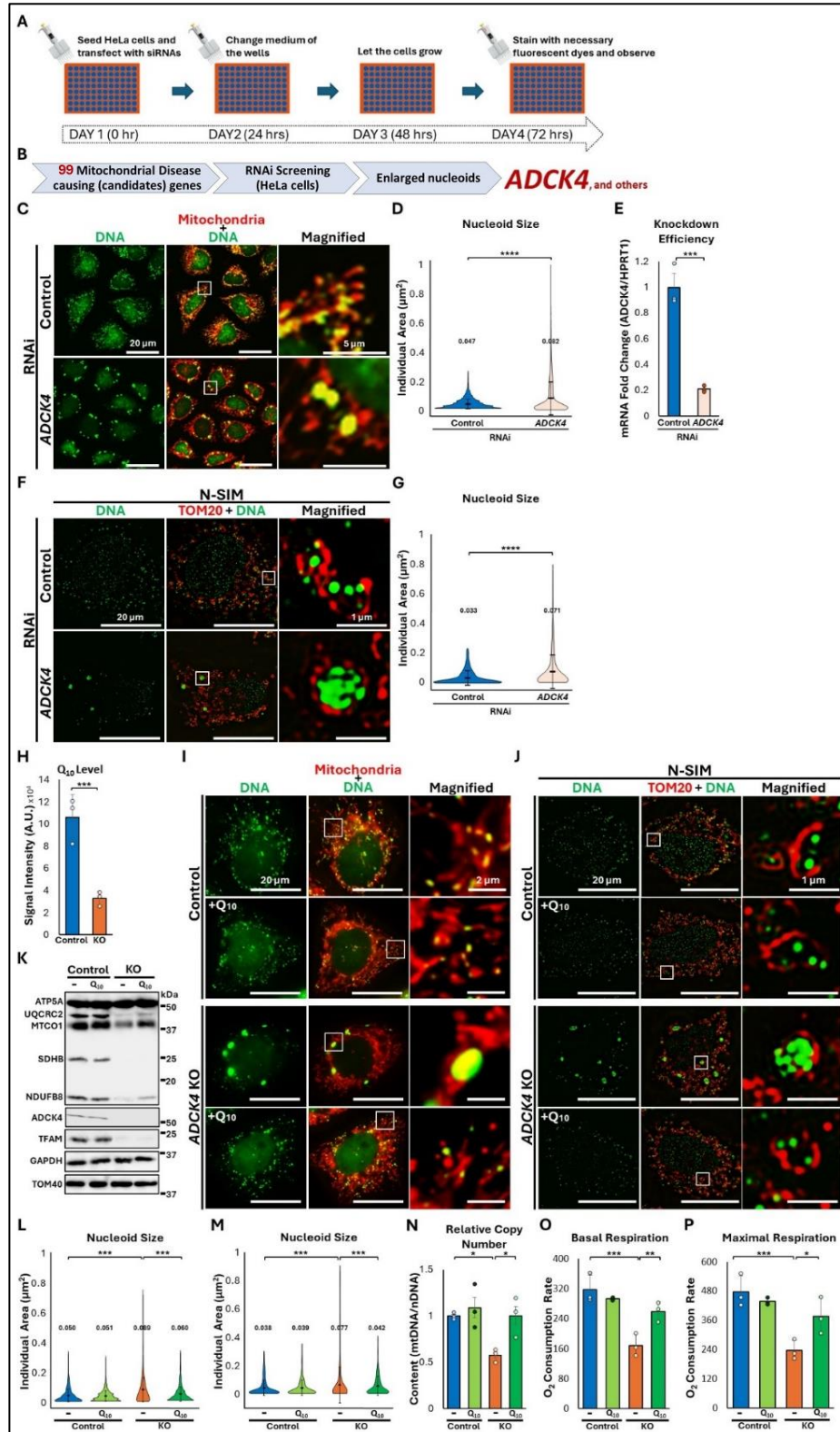


Fig 2. ADCK4 affects mitochondrial nucleoid morphology and function via ubiquinone biosynthesis. A))

RNAi screening protocol on HeLa cells. B) Stepwise filtering of candidates via fluorescence microscopy. C) Fluorescence microscopy images of *ADCK4* RNAi cells (mitochondria, red; nucleoids, green) under 20x magnification (scale bar 20 μm , magnified scale bar 5 μm). Graphs showing the D) nucleoid size (n=more than 50 cells), and E) knockdown efficiency comparison with a control set (n=3 independent cell sets). F) Fluorescence super resolution microscopy images via immunostaining of *ADCK4* RNAi cells (TOM20, red; nucleoids, green) under 100x magnification (scale bar 20 μm , magnified scale bar 1 μm). G) Graph showing the nucleoid size obtained from super resolution microscopy (n=3 cells). H) Graph showing ubiquinone (Q_{10}) levels measured via LC-MS in control and KO cells (n=3 independent cell sets). Q_{10} treatment in KO cells followed by I) fluorescence microscopy (mitochondria, red; nucleoids, green) under 60x magnification (scale bar 20 μm , magnified scale bar 2 μm), J) super resolution microscopy via immunostaining (TOM20, red; nucleoids, green) under 100x magnification (scale bar 20 μm , magnified scale bar 1 μm), K) immunoblotting of respiratory chain proteins, TFAM and *ADCK4*, quantification of nucleoid sizes from L) fluorescence microscopy (n=more than 50 cells) and M) super resolution microscopy (n=3 cells), measurement of N) mtDNA content by qPCR (n=3 independent cell sets), O) basal and P) maximal respiration rates (pmol/min/total protein; n=3 independent cell sets) by extracellular flux analysis in comparison to control cells. Error bars are shown in standard deviation values. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$; where P values were determined by D,E,G) two-sided Student's *t*, H) Kruskal-Wallis, L-P) one-way ANOVA and Tukey-Kramer tests. D,G,L,M) Decimal numbers indicate the mean values of different populations.

I particularly focussed on *ADCK4* (Aarf Domain-Containing Kinase 4) /*COQ8B*; a factor which plays a specific, yet uncharacterized, role in ubiquinone biosynthesis inside the mitochondrial inner membrane. I confirmed that the RNAi of *ADCK4* resulted in increment of the nucleoid puncta size (Fig 2C,D) along with severe repression of *ADCK4* mRNA (Fig 2E). Given the limitations of conventional fluorescence microscopy in terms of resolving capacity³⁸, I checked for the morphological changes of nucleoids via super-resolution microscopy (N-SIM) as well (Fig 2F,G), and it showed the same trend of nucleoid enlargement in *ADCK4* knockdown cells (average area is around 0.07-0.09 μm^2 in *ADCK4* KO cells, and around 0.03-0.05 μm^2 in control cells). The *ADCK4* RNAi led to the alteration of only the nucleoid morphology, but not the mitochondrial length (Fig 3A,B). Thus, I found *ADCK4* as a novel regulator of nucleoid structure.

ADCK4 mediated ubiquinone biosynthesis is required for maintaining mitochondrial nucleoid morphology and respiratory complexes

It has been reported that mutations in *ADCK4* gene causes mitochondrial disease with severe glomerulopathy^{39,40}. Mutations in *ADCK4* causes primary coenzyme Q_{10} shortage⁴¹, and by supplementing ubiquinone, the pathogenesis in *ADCK4* patients were reported to be rescued, suggesting that loss of ubiquinone causes mitochondrial dysfunction^{39,42}.

To illuminate the impact of *ADCK4* on nucleoid morphology, I established an *ADCK4* knockout (KO) HeLa cell line using CRISPR/Cas9 system (Fig 4A) and confirmed complete repression of the *ADCK4* protein (Fig 2K). As expected, I observed that nucleoids were significantly enlarged in *ADCK4* KO cells by both conventional fluorescent (Fig 2I,L) and super-resolution (Fig 2J,M) microscopy in comparison to the control set. It was previously showed in mitochondrial fission-deficient cells that

altering mitochondrial nucleoid morphology and distribution affected the mitochondrial respiratory chain function; that was an outcome of defective mitochondrial membrane dynamics^{28,30}. Although mitochondrial length was not affected in the ADCK4 KO cells, similar to the ADCK4 knockdown cells (Fig 3A,B), electron microscopy showed the cristae structures to be slightly impaired (Fig 3C,D). These results suggested that nucleoid enlargement is not caused by imbalanced mitochondrial fusion and fission in the ADCK4 deficient cells, unlike mitochondrial fission deficient cells²⁹.

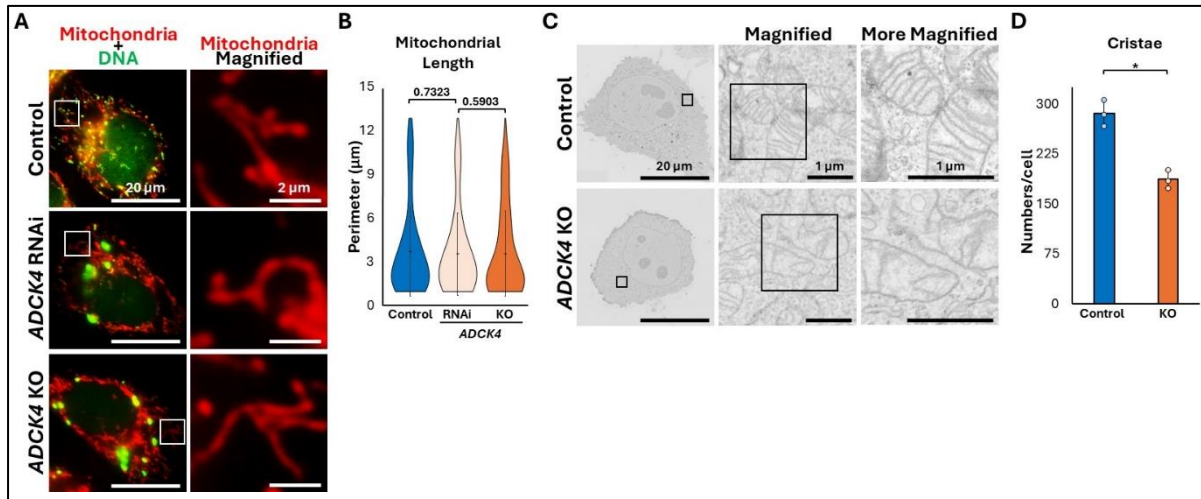


Fig 3. Morphometric analysis of mitochondria in *ADCK4* deficient cells. A) Fluorescence microscopy images of *ADCK4* RNAi and KO cells (mitochondria, red; nucleoids, green) under 60x magnification (scale bar 20 μm , magnified scale bar 2 μm). B) Graph showing the mitochondrial length (n=more than 10 cells), with respect to a control set. C) Electron microscopy of showing cristae structures of the mitochondria in KO cells (scale bar 20 μm , magnified scale bars 1 μm). D) Graph showing cristae number (n=3 cells), with respect to a control set. Error bars are shown in standard deviation values. * $P < 0.05$; where P values were determined by B) two-sided Student's *t* and D) one-way ANOVA and Tukey-Kramer tests.

Next, I examined the effects of ADCK4 repression on mitochondrial respiratory function. When I analysed oxygen consumption rate (OCR), both basal and maximal respiration were significantly reduced in ADCK4 KO cells (Fig 2O,P); as expected because ADCK4 is a mitochondrial disease causing gene. Since ADCK4 is a critical factor in ubiquinone biosynthesis pathway, levels of ubiquinone were diminished in the patient cells⁴³. In my ADCK4 deficient HeLa cells, a significant reduction of Q₁₀ levels was observed (Fig 2H). To evaluate the role of ubiquinone, I applied ubiquinone to ADCK4 deficient cells. As expected, the reduced rates of basal and maximal respiration were brought back to their normal states (Fig 2O,P). I also found the restoration of the enlarged nucleoids back to their normal state (Fig 2I,J,L,M), indicating the critical role of ubiquinone in nucleoid morphological regulation.

It is well known that ubiquinone is essential for electron transport and antioxidation in mitochondria. However, I unexpectedly found subunits of respiratory chain I (NDUFB8), III (UQCRC2), and IV (MTCO1) to be severely reduced in the ADCK4 deficient cells (Fig 2K), and they were rescued by adding ubiquinone. Similar reductions of respiratory chain subunits and their corresponding rescue via nucleoid restoration to

normal state by ubiquinone treatment was also observed in COQ3 and COQ7 knockdown cells (Fig 5E,F). Furthermore, TFAM protein level (Fig 2K) and mtDNA content (Fig 2N) were also reduced in the ADCK4 deficient cells. Ubiquinone treatment rescued the reductions of mtDNA content (Fig 2N), suggesting that ubiquinone is critical for the mtDNA function. However, the reduced TFAM was not rescued by the added ubiquinone (Fig 2K), suggesting that enlarged nucleoids and mtDNA dysfunction are not caused by the reduction of TFAM, and the remaining TFAM might have enough function for the nucleoid formation in this condition. These data indicated that the nucleoid morphology and respiratory complex formation were affected by loss of ubiquinone in the ADCK4 deficient cells.

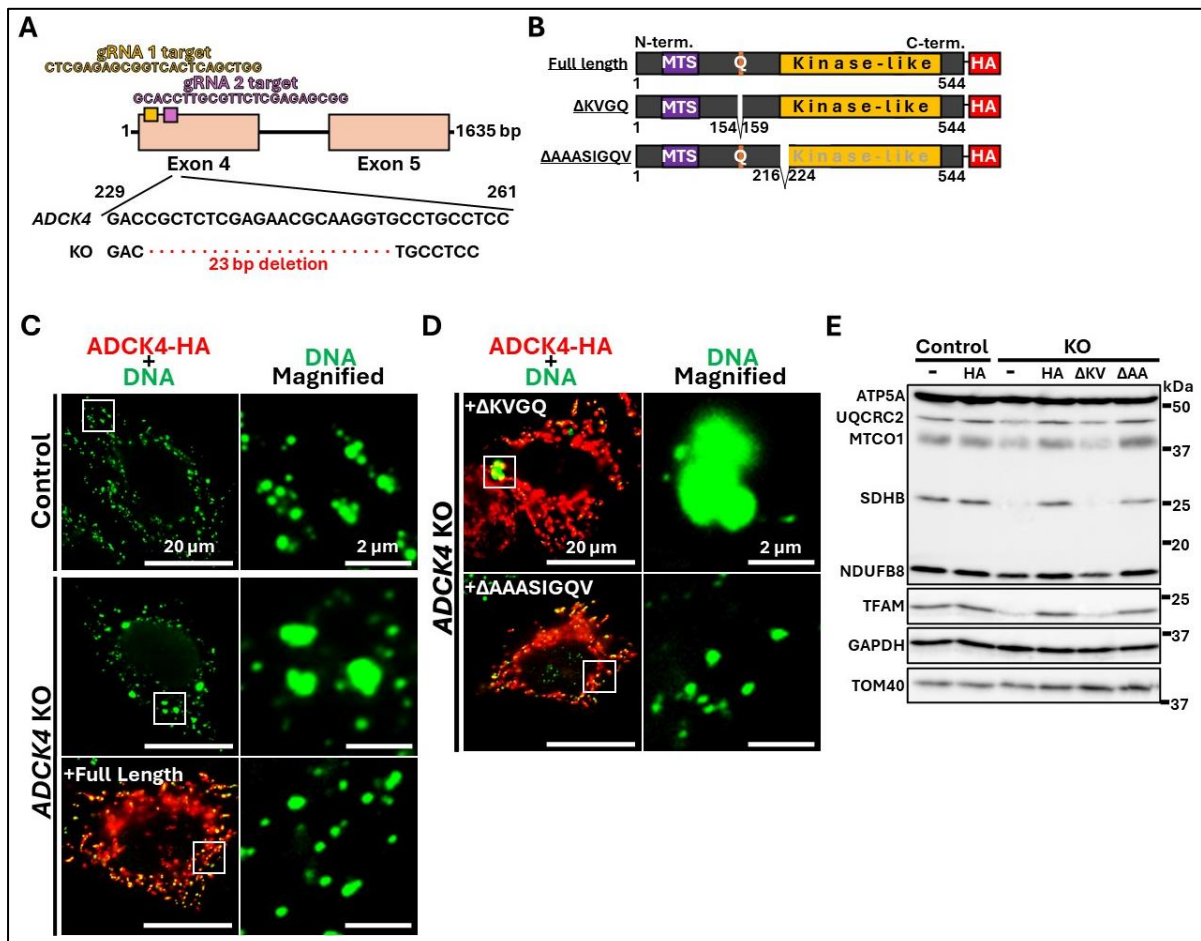


Fig 4. Establishment of *ADCK4* knock out (KO) cells and subsequent rescue experiments. A) CRISPR/Cas9 target site for guide RNAs in generating *ADCK4* knock out cells along with the sequence alignments. B) HA-tagged full length and truncated versions of ADCK4 used in rescue experiments. C,D) Fluorescence microscopy images via immunostaining of rescue experiments using B) (ADCK4-HA, red; nucleoids, green) under 60x magnification (scale bar 20 μm, magnified scale bar 2 μm). E) Immunoblots of respiratory chain proteins and TFAM in rescue experiments with ADCK4-HA full (HA), ΔKVGQ (ΔKV) and ΔAAASIGQV (ΔAA) constructs on KO cells.

Rescue experiments by re-introducing the full-length exogenous ADCK4 completely restored the nucleoid morphology as seen in control cells (Fig 4C), although KxGQ motif deletion mutant (ΔKVGQ), responsible for aiding ubiquinone biosynthesis⁴⁴, failed to rescue the nucleoid morphology (Fig 4B,D). It was also reported that ADCK4

has kinase-like motif, and thus it was believed to have kinase activity; however, the specific substrates and products for the potential kinase activity are not well known⁴⁵. Here I found that the kinase-like motif deletion mutant (Δ AAASIGQV) could rescue the nucleoid morphology in the ADCK4 KO cells, suggesting that ADCK4 regulates nucleoid morphology, but not as a kinase. A remarkable loss of respiratory chain subunits of complexes I, III, and IV was rescued by re-introduction of full-length or kinase-like motif mutant (Δ AAASIGQV) of ADCK4 but not by the KxGQ motif mutant (Fig 4E), thereby showing that ubiquinone biosynthesis mediated by ADCK4 is essential for the regulation of respiratory complex formation. Unexpectedly, complex II, which is composed from only nuclear genome encoded subunits, was also repressed in ADCK4 KO cells. It was rescued by re-introduction of full-length or kinase-like motif mutant, but not rescued by adding ubiquinone (Fig 2K, Fig 4E), thereby suggesting that the phenotype on complex II is dependent on ADCK4 but not on ubiquinone biosynthesis directly. Although it is contradictory that the ubiquinone biosynthesis mutant could not rescue complex II subunits in the ADCK4 KO cells (Fig 4E) by an unknown reason, further analysis is needed regarding the role of nucleoids and ubiquinone on complex II. Nonetheless, these data clearly showed that ubiquinone biosynthesis, mediated by ADCK4, is essential for nucleoid morphology as well as for the formation of respiratory complexes I, III and IV.

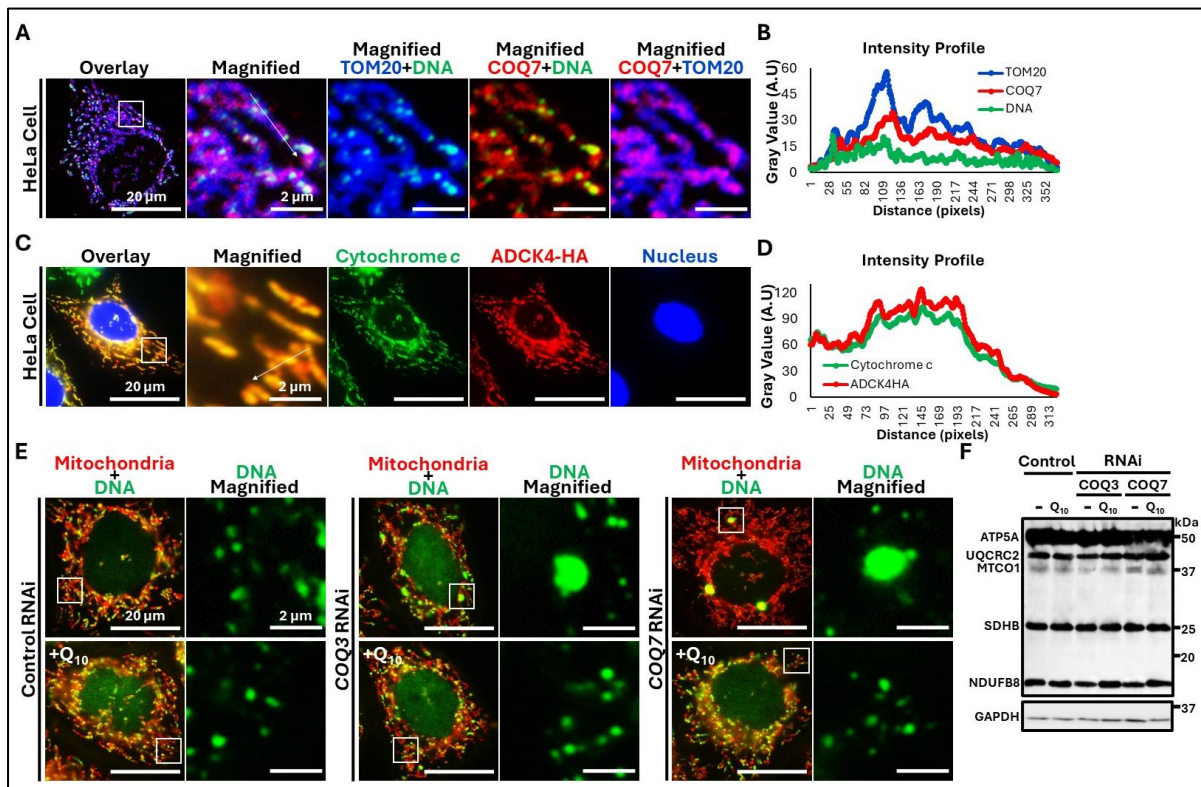


Fig 5. Relationship between ubiquinone biosynthetic complex members and mitochondrial nucleoids in HeLa cells. A) Fluorescence microscopy image of a representative HeLa cell (TOM20, blue; nucleoids, green, COQ7, red) under 60x magnification (scale bar 20 μ m, magnified scale bar 2 μ m) and graph showing B) intensity plot for endogenous COQ7 distribution with respect to mitochondria and nucleoids. C) Fluorescence microscopy image of a representative HeLa cell (Cytochrome c, green; ADCK4-HA, red; nucleus, blue) under 60x magnification (scale bar 20 μ m, magnified scale bar 5 μ m) and graph showing D) intensity plot for Cytochrome c and ADCK4-HA. E) Fluorescence microscopy images of Control RNAi and COQ3 RNAi cells with Mitochondria (red), DNA (green), and Magnified views. F) Western blot showing protein levels of ATP5A, UQCRC2, MTCO1, SDHB, NDUFB8, and GAPDH in Control and COQ3 RNAi cells.

showing D) intensity plot for exogenous ADCK4 distribution in mitochondria. White arrows indicate zones of measurement. Q₁₀ treatment in COQ3 and COQ7 RNAi cells followed by E) fluorescence microscopy (mitochondria, red; nucleoids, green) under 60x magnification (scale bar 20 μ m, magnified scale bar 2 μ m), F) immunoblotting of respiratory chain proteins in comparison to a control set.

Ubiquinone maintains nucleoid structure irrespective of its traditional functions

Ubiquinone is traditionally known to have two major functions – one as an electron carrier in the electron transport chain, and the other as an antioxidant (Fig 6A). To examine whether the electron transferring property of ubiquinone is responsible for maintaining the nucleoid structures, I applied various quinone family molecules to ADCK4 deficient cells and checked these effects on nucleoids and respiration. Menaquinone-4 (MK-4) is a quinone molecule, a major component of vitamin K₂ and acts in the bacterial electron transport chain. Similar to the ubiquinone, menaquinone-4 is an antioxidant, however, it cannot function as an electron carrier in mammalian mitochondria, due to incompatible oxidation-reduction potential in the electron transport chain in mitochondria⁴⁶. I found that menaquinone-4 treatment successfully restored the nucleoid structures (Fig 6B,C), and also partly rescued the reduced levels of respiratory chain subunits (Fig 6F,G,H), as seen in ubiquinone treated cells. Also similar to ubiquinone (Fig 2K), menaquinone-4 did not rescue complex II subunit, TFAM and ADCK4 protein levels (Fig 6F). However, menaquinone-4 failed to rescue both basal and maximal respiratory activities (Fig 6D,E), unlike ubiquinone, which is in line with the previous report of menaquinone-4 not being able to compensate the loss of ubiquinone as an electron carrier⁴⁶. I also examined other quinone molecules and found that phyloquinone (PhQ)/vitamin K₁, found in photosynthetic organisms, also mediates nucleoid structure (Fig 6M,N). I further showed that neither respiratory complex inhibitors, such as rotenone (complex I inhibitor), carboxin (complex II inhibitor) and antimycin A (complex III inhibitor), nor respiratory chain subunit SDHD knockout affected nucleoid structures (Fig 7A-C). These results suggested that electron transferring property of ubiquinone as well as the electron transport chain are not responsible for maintaining the nucleoid structures.

Next, to check whether the generation of reactive oxygen species (ROS) and the subsequent antioxidizing function of ubiquinone affects nucleoid structure, I treated ADCK4 KO cells with MitoTEMPO, a well-known mitochondrial superoxide scavenger (Fig 6A). The moderate amounts of reactive oxygen species, generated in ADCK4 KO cells, were quenched by both ubiquinone and MitoTEMPO as confirmed by fluorescence microscopy (Fig 6K; Fig 7D,F) and LC-MS analyses (Fig 6L; Fig 8A-C). LC-MS analyses is a more accurate way of measuring reactive oxygen species production via quantifying the levels of 2-Hydroxyethidium, a superoxide-specific oxidation product⁴⁷. However, only ubiquinone rescued the enlarged nucleoids back to normal (Fig 6I,J) and also restored the lowered mitochondrial membrane potential (Fig 7E,G), suggesting that antioxidizing function of ubiquinone is not critical for affecting nucleoid structures. These results clearly showed that biological quinones

possess a novel function of maintaining nucleoid structures and respiratory chain complexes, which is unrelated to their traditional functions.

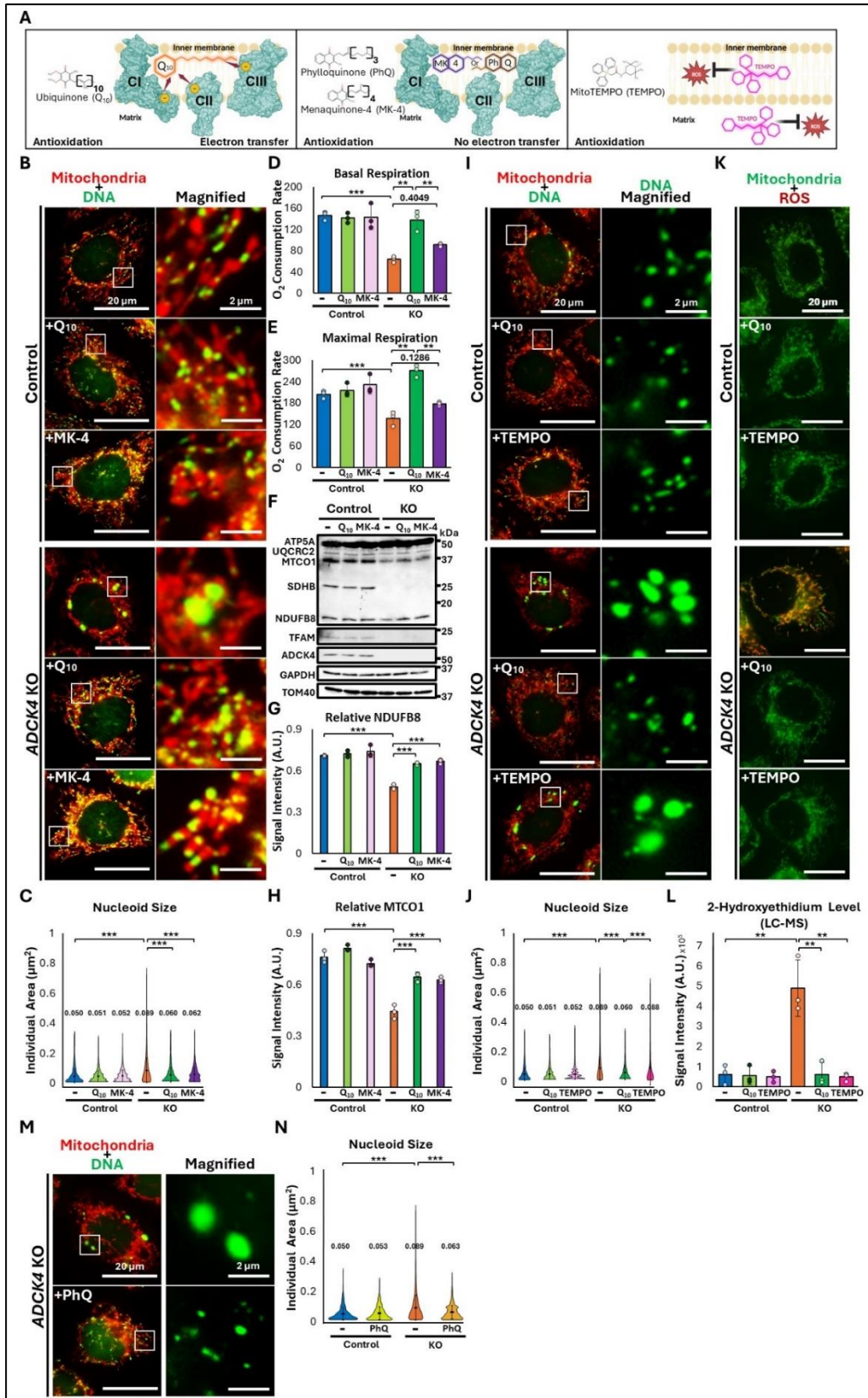


Fig 6. Natural quinones can govern mitochondrial nucleoid structure and stability of respiratory chain subunits regardless of their traditional functions. A) Graphical representation of Q₁₀, menaquinone-4 (MK-4), phylloquinone (PhQ) and Mito-TEMPO (TEMPO) roles in mammalian mitochondria. Q₁₀ or MK-

4 treatment in KO cells followed by B) fluorescence microscopy (mitochondria, red; nucleoids, green) under 60x magnification (scale bar 20 μ m, magnified scale bar 2 μ m), quantification of C) nucleoid sizes (n=more than 50 cells), D) basal & E) maximal respiration rates (pmol/min/total protein; n=3 independent cell sets) as analysed by extracellular flux analysis, F) immunoblots of respiratory chain proteins, TFAM and ADCK4 in comparison to control cells and quantification of relative band intensities in the immunoblots of G) NDUFB8 and H) MTCO1. Q₁₀ or TEMPO treatment in KO cells followed by fluorescence microscopy I) (mitochondria, red; nucleoids, green), K) (mitochondria, green; reactive oxygen species/ROS, red) under 60x magnification (scale bar 20 μ m, magnified scale bar 2 μ m) and quantification of J) nucleoid sizes (n=more than 50 cells) and L) 2-Hydroxyethidium levels via LC-MS (n=3 independent cell sets) in comparison to control cells. Phylloquinone (PhQ) treatment in KO cells followed by M) fluorescence microscopy (mitochondria, red; nucleoids, green) under 60x magnification (scale bar 20 μ m, magnified scale bar 2 μ m) and N) quantification of nucleoid sizes (n=more than 50 cells). Error bars are shown in standard deviation values. *** P <0.001 and ** P <0.01; where P values were determined by C-E,G,H,J,L,N) one-way ANOVA and Tukey-Kramer tests. C,J,N) Decimal numbers indicate the mean values of different populations.

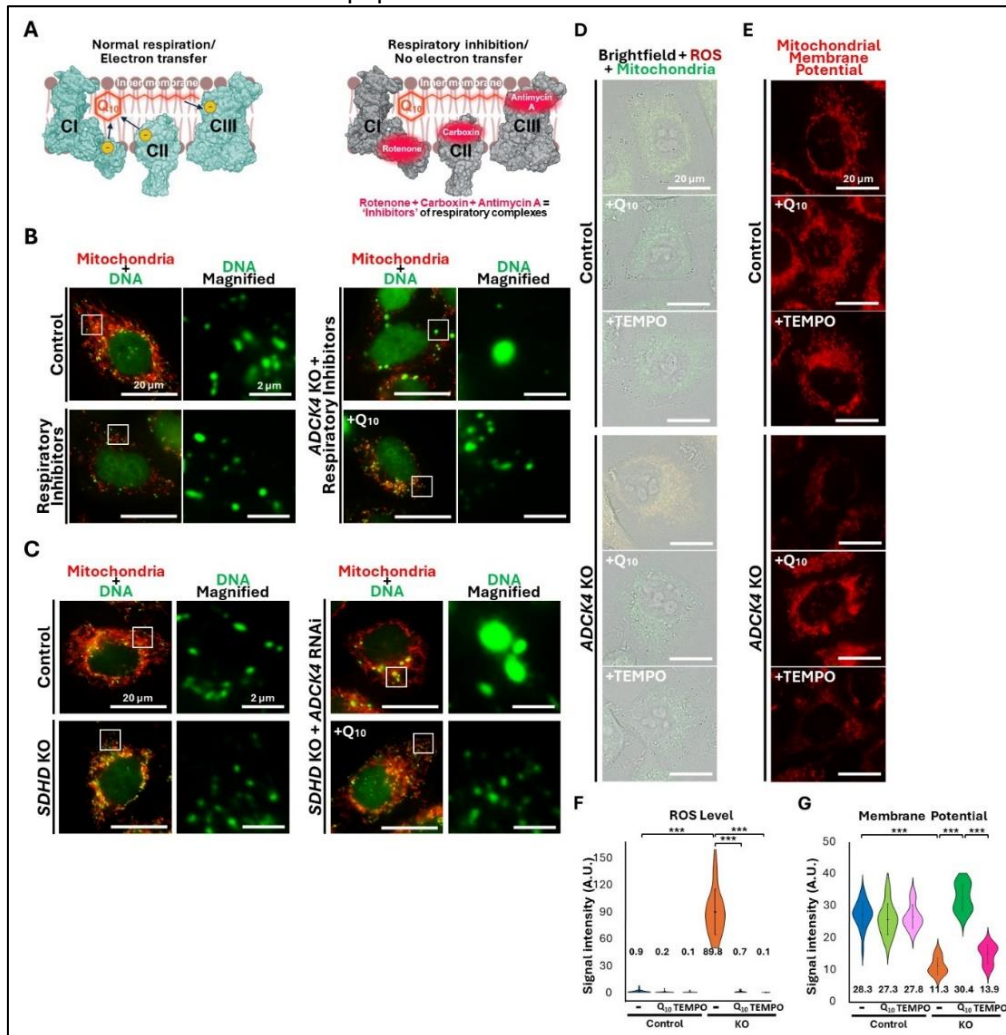


Fig 7. Effects of respiratory chain defects and botanical quinone on mitochondrial nucleoids. A) Graphical representation of the roles of Rotenone, Carboxin and Antimycin A (Inhibitors) in mammalian mitochondria. Q₁₀ treatment in B) *ADCK4* KO cells with or without application of inhibitors, C) *SDHD* knock out (*SDHD* KO) cells along with *ADCK4* RNAi followed by fluorescence microscopy (mitochondria, red; nucleoids, green) under 60x magnification (scale bar 20 μ m, magnified scale bar 2 μ m). D) Brightfield merged images from Fig 2K showing the cell boundary and E) fluorescent microscopy images of an identical set to determine mitochondrial membrane potential (mitochondria/TMRM, red)

under 60x magnification (scale bar 20 μm , magnified scale bar 2 μm), followed by quantification of F) ROS signal intensities (n =about 20 cells) and G) mitochondrial membrane potential/TMRM intensities (n =20 cells) in comparison to control cells. Error bars are shown in standard deviation values. *** P <0.001; where P values were determined by F,G) one-way ANOVA and Tukey-Kramer tests. F,G) Decimal numbers indicate the mean values of different populations.

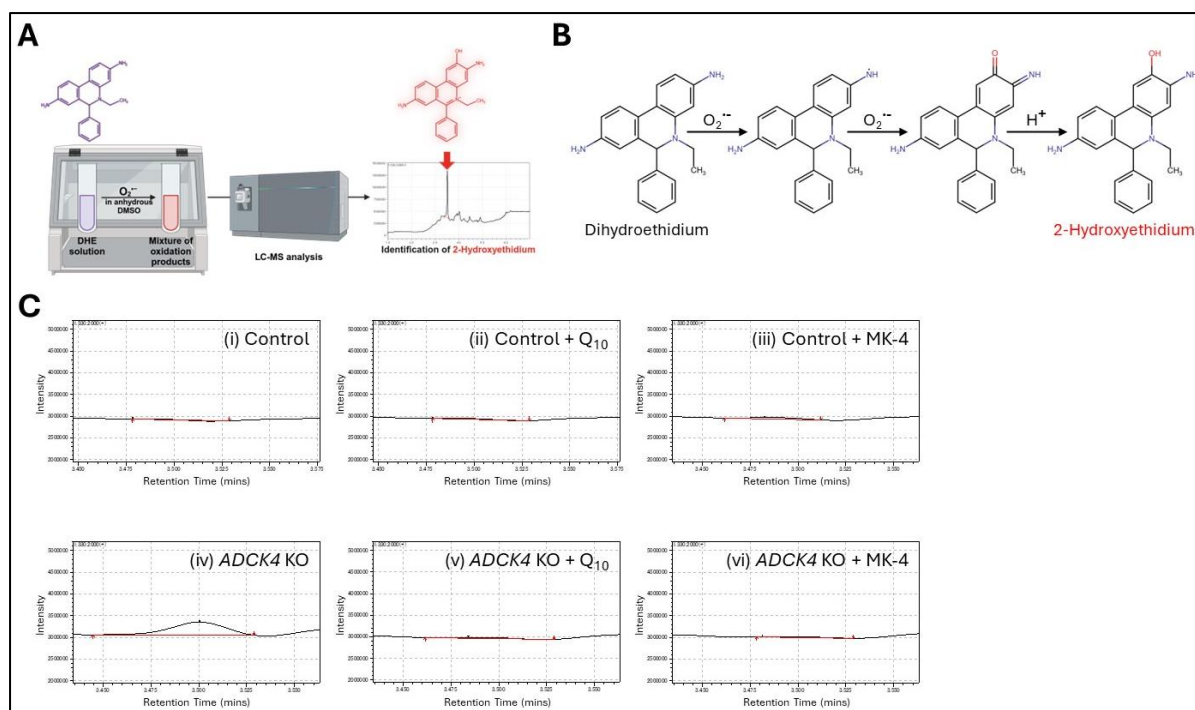


Fig 8. Synthesis of 2-Hydroxyethidium standard and using it subsequent LC-MS analysis. A) Graphical representation of synthesis of 2-Hydroxyethidium standard from dihydroethidium (DHE) followed by its purification and characterization via LC-MS. B) Reaction mechanism for conversion of DHE to 2-Hydroxyethidium. C) Representative graphs showing the presence or absence of 2-Hydroxyethidium ions detected at m/z 330.2 in i) control, ii) control + Q_{10} , iii) control + MK-4, iv) *ADCK4* KO, v) *ADCK4* KO + Q_{10} and vi) *ADCK4* KO + MK-4 cell sets. The red lines indicate the baseline.

Ubiquinone mediates nucleoid formation in cooperation with cardiolipin in inner membrane

To analyse the detailed function of ubiquinone in nucleoid formation, I reconstituted nucleoid structures *in vitro* using a circular DNA⁴⁸ and TFAM protein which is known to be essential for mtDNA packaging and proper nucleoid formation^{21,22}. Here I observed the reconstituted nucleoid structures via fluorescent microscopy that was stained by SYBR Green I and confirmed that TFAM mediates formation of dot-like nucleoid structures (Fig 9A-a,B), but ubiquinone, instead of TFAM, did not mediate nucleoid formation (Fig 9A-b,B).

In a system where DNA was mixed with ubiquinone in the presence of liposomes containing 25% molar ratio of cardiolipin ("CL-25" liposomes)⁴⁹, mimicking the mitochondrial inner membrane along with TFAM, two distinct kinds of nucleoid structures were formed (Fig 9C-d,D,E) – one in an annular fashion around the liposomal membrane, and the other in a dispersed and membrane-independent fashion. This suggested that the combination of ubiquinone and cardiolipin in the

presence of TFAM mediates nucleoid formation and its membrane association. When either cardiolipin or ubiquinone was removed from this system, only the dispersed and membrane-free nucleoids remained (Fig 9C-a,-b,D) as seen in Fig 9A-a, suggesting that ubiquinone supports membrane binding of nucleoids. However, when the CL-25 liposomes were replaced with liposomes having 4% molar ratio of cardiolipin (“CL-4” liposomes)⁴⁹, mimicking the mitochondrial outer membrane, once again only the dispersed and membrane-free nucleoids remained, irrespective of the absence or presence of ubiquinone (Fig 9C-c,-e,D). This indicated that ubiquinone mediates nucleoid formation and its association with the inner membrane containing sufficient cardiolipin (schematic model in Fig 9F).

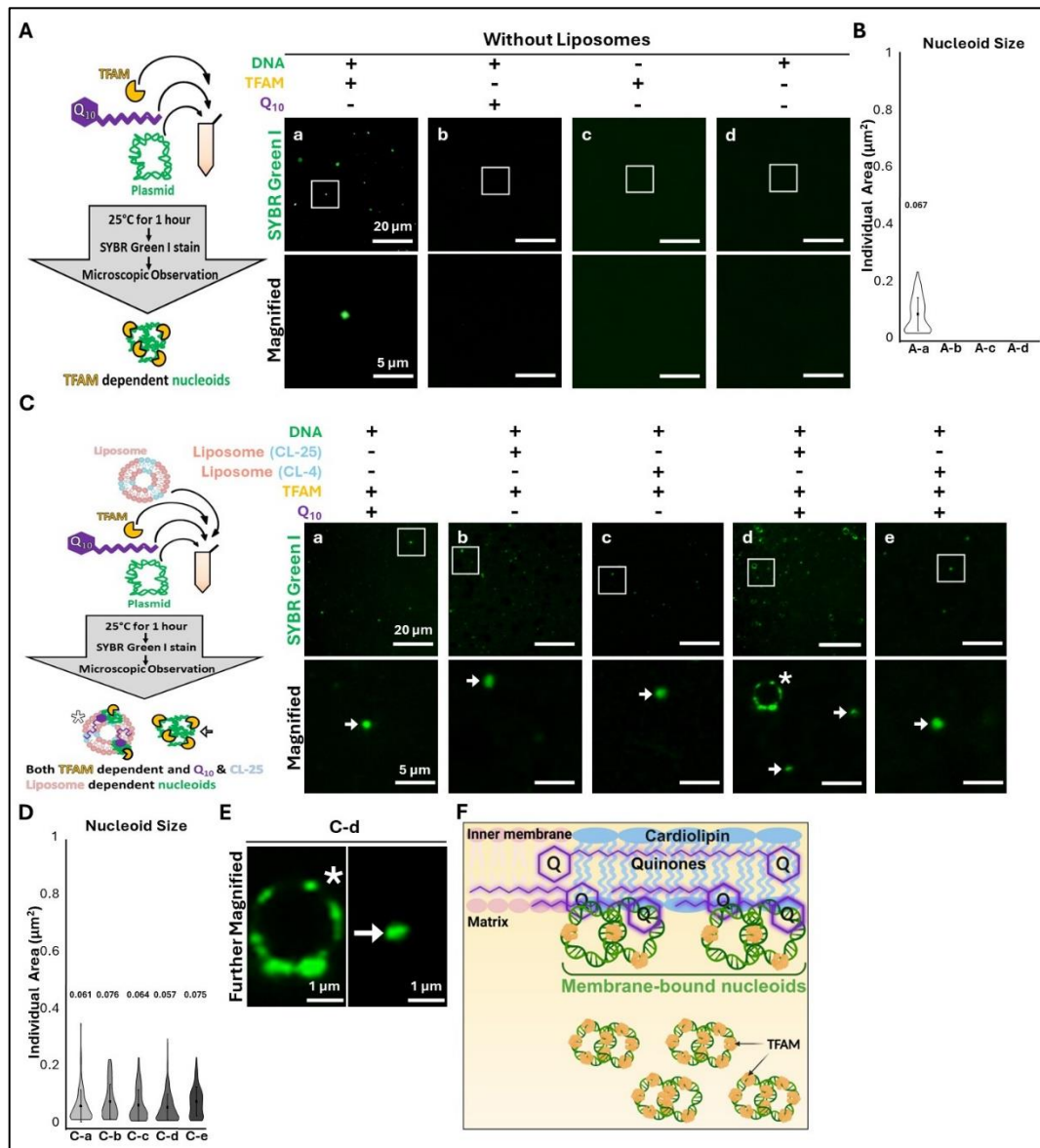


Fig 9. *In vitro* nucleoid formation in a TFAM and liposome dependent manner. Circular DNA (green) only detected when nucleoid like structures were formed. Fluorescence microscopy images of nucleoid like structures formed after A) incubating circular DNA with purified His₆-TFAM or Q₁₀ and C) introduction of mitochondrial inner membrane mimicking CL-25 or outer membrane mimicking CL-4 liposomes in the mix along with necessary controls and respective graphical representations of the workflow. B) and D) Graphs showing quantified data of nucleoid sizes formed in A) and C) respectively (n=more than 100

nucleoids). E) Expanded view nucleoids formed in C-d) in annular (asterisk) and dispersed (arrow) manners. F) Proposed graphical representation of ‘membrane-bound’ quinone and TFAM-based and ‘free’ only TFAM-based nucleoids in mammalian cells. Error bars are shown in standard deviation values. B,D) Decimal numbers indicate the mean values of different populations.

In this analysis, I also found dot-like structures similar to nucleoids, even in the absence of TFAM (Fig 10A-a,B). However, in the absence of both ubiquinone and TFAM, broad signals were observed instead of the dot-like structures, which might be non-specific binding of DNA or SYBR Green I on the surface of the CL-25 liposomes (Fig 10A-b,B). Also, no nucleoid-like structures were observed when CL-25 liposomes were replaced with CL-4 liposomes (Fig 10A-c,-d,B). I validated this finding of peculiar nucleoid-like structures in cultured cells and showed that adding excess ubiquinone was able to partially rescue enlarged nucleoids back to their smaller and dispersed state in TFAM-repressed cells²¹ (Fig 10C,D). However, the effect of ubiquinone on nucleoids was mild and not enough to rescue the mtDNA content (Fig 10E), thereby further underlining the significance of TFAM in maintaining mtDNA^{50,51}, although I found that ubiquinone should support nucleoid morphology in the TFAM deficient condition.

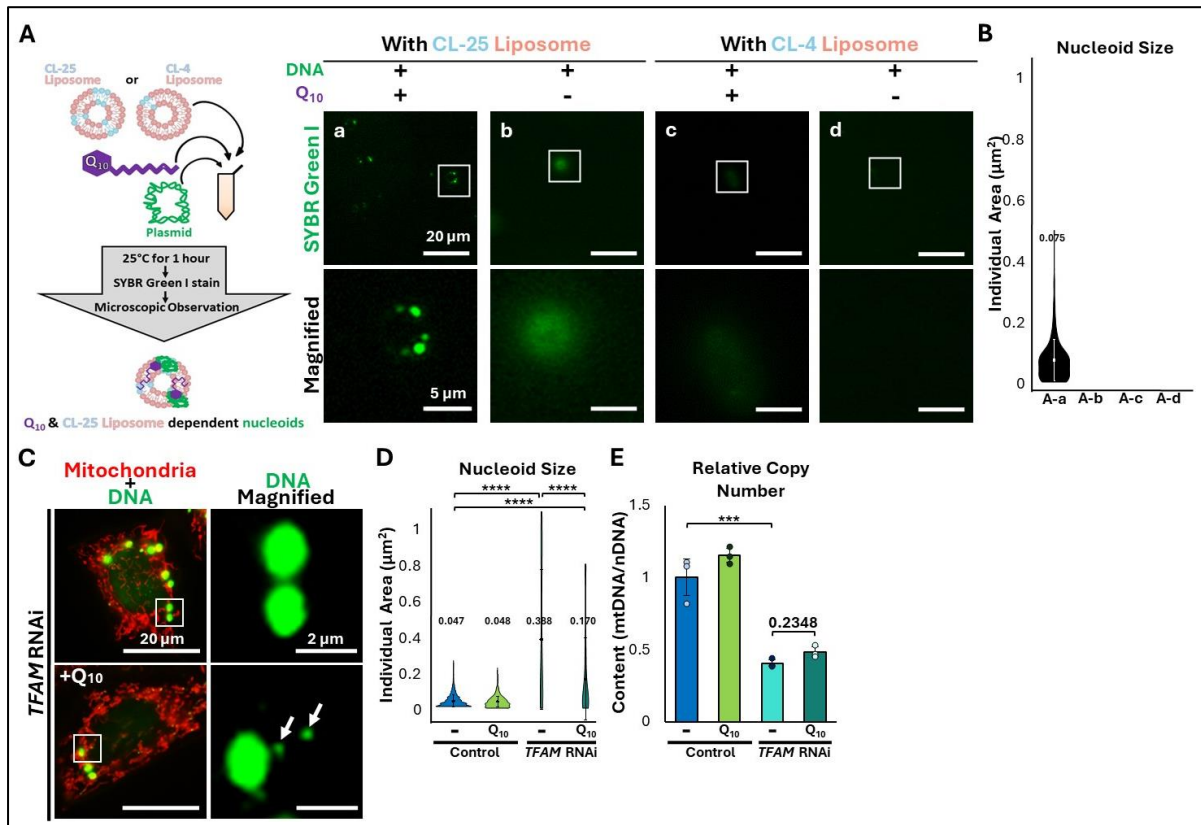


Fig 10. Formation of nucleoid like structures in a TFAM-independent manner. A) *In vitro* analysis showing circular DNA (green) only detected when nucleoid like structures were formed. Fluorescence microscopy images of nucleoid like structures formed after addition of mitochondrial inner membrane mimicking CL-25 or outer membrane mimicking CL-4 liposomes to circular DNA and Q₁₀ incubated samples along with necessary controls and graphical representation of the workflow. B) Graph showing quantified data of nucleoid sizes formed in A) (n=more than 100 nucleoids). C) Q₁₀ treatment in TFAM knock down cells followed by fluorescence microscopy (mitochondria, red; nucleoids, green; white arrows, small nucleoids) under 60x magnification (scale bar 20 μm, magnified scale bar 2 μm), D)

quantification of nucleoid sizes (n =more than 50 cells) and E) measurement of mtDNA content by qPCR (n =3 independent cell sets) in comparison to control cells. Error bars are shown in standard deviation values. **** P <0.0001 and *** P <0.001; where P values were determined by D,E) one-way ANOVA and Tukey-Kramer tests. B,D) Decimal numbers indicate the mean values of different populations.

Quinones protect mitochondrial function from toxicity of doxorubicin by maintaining nucleoid morphology

As shown above, I found that adding excess ubiquinone could rescue the enlarged nucleoids formed in ADCK4 KO cells. In order to utilize this quinone-based nucleoid structural maintenance, I developed a new method to improve mitochondrial function under a pathological condition caused by the anti-cancer drug doxorubicin, which causes severe cardiotoxicity as a side effect⁵². In addition, doxorubicin has been reported to intercalate with mtDNA and deform nucleoid structures⁵³, which should cause mitochondrial dysfunction. I confirmed that doxorubicin treatment led to nucleoid enlargement (Fig 11A-C), along with the drastic reduction of basal and maximal respiratory rates (Fig 11D,E). Then I checked the effect of ubiquinone. When cells were incubated with exogenous ubiquinone before the doxorubicin-treatment, both nucleoid enlargement and respiratory failure were almost completely repressed, as in doxorubicin non-treated cells (Fig 11A-E). However, instead of pre-treatment, when ubiquinone application was done after doxorubicin application, it failed to resist nucleoid enlargement and respiratory dysfunction (Fig 11A-E).

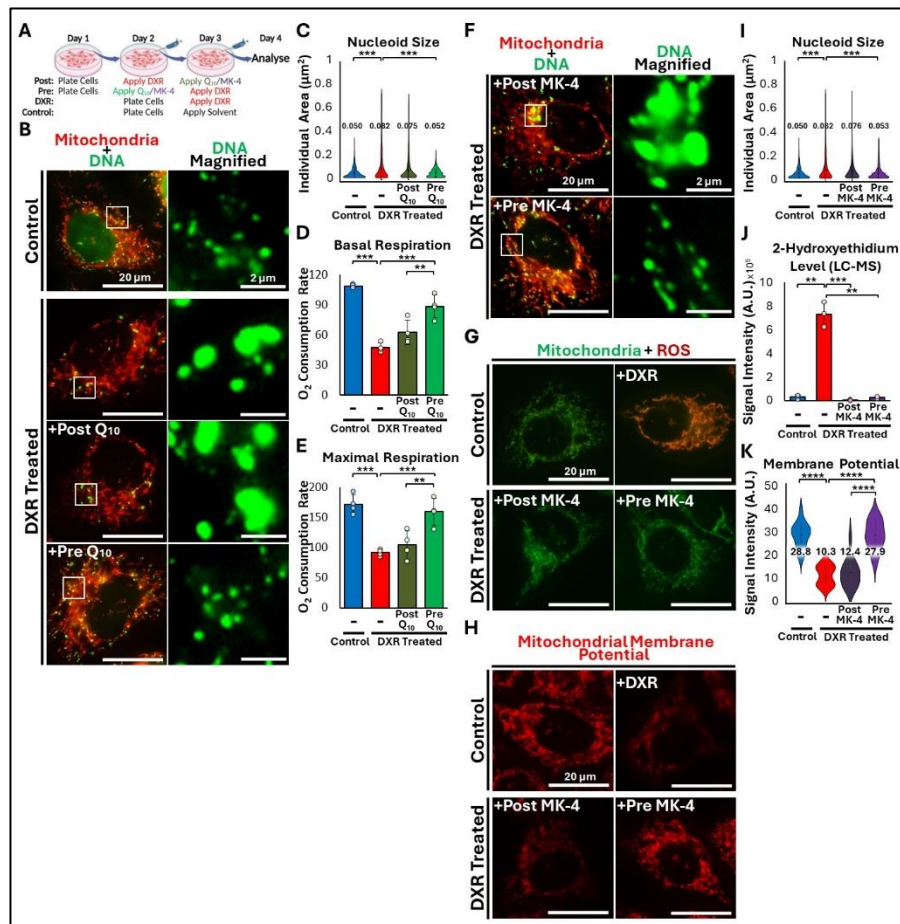


Fig 11. Pre-treatment with quinones resist doxorubicin (DXR) induced mitochondrial dysfunction via

regulating nucleoids. A) Graphical representation of experimental model. DXR treated HeLa cells subjected to pre or post-treatment of Q₁₀ followed by B) fluorescence microscopy (mitochondria, red; nucleoids, green) under 60x magnification (scale bar 20 μ m, magnified scale bar 2 μ m), quantification of C) nucleoid sizes (n=more than 30 cells), D) basal and E) maximal respiration rates (pmol/min/total protein; n=4 independent cell sets) as analysed by extracellular flux analysis in comparison to control cells. DXR treated HeLa cells subjected to pre or post-treatment of MK-4 followed by fluorescence microscopy F) (mitochondria, red; nucleoids, green), G) (mitochondria, green; reactive oxygen species/ROS, red), H) (mitochondria/TMRM, red) under 60x magnification (scale bar 20 μ m, magnified scale bar 2 μ m) and quantification of I) nucleoid sizes (n=more than 50 cells), J) 2-Hydroxyethidium levels via LC-MS (n=3 independent cell sets) and K) mitochondrial membrane potential/TMRM intensities (n=20 cells) in comparison to control cells. Error bars are shown in standard deviation values. **** P <0.0001, *** P <0.001 and ** P <0.01; where P values were determined by C-E,I-K) one-way ANOVA and Tukey-Kramer tests. C,I,K) Decimal numbers indicate the mean values of different populations.

Similar experiments with menaquinone-4 showed that pre-treatment, but not post-treatment, could resist doxorubicin dependent nucleoid enlargement (Fig 11F,I) and lowering of mitochondrial membrane potential (Fig 11H,K). Furthermore, I checked the antioxidizing capacity of menaquinone-4 and found that even post-treatment of menaquinone-4 could sufficiently quench the reactive oxygen species formed due to doxorubicin via fluorescence microscopy (Fig 11G; Fig 12A,B) and LC-MS analyses of 2-Hydroxyethidium levels (Fig 11J; Fig 12C). With investigations being done on nucleoid activity under the influence of DNA intercalators⁵⁴, I then decided to check if this quinone-based nucleoid structural maintenance also affects it. Chasing experiments with 5-ethynyl-2'-deoxyuridine (EdU) showed that although under doxorubicin exposure, the replication of nuclear DNA and mtDNA in enlarged nucleoids were halted, pre-treatment with either ubiquinone or menaquinone-4 resumed only the activity of mtDNA replication from within the dispersed nucleoids (Fig 12D,E). This further emphasized that generation of reactive oxygen species is unrelated to nucleoid structural maintenance and also showed that the added excess quinones do not deactivate the main effect of doxorubicin on the nuclear DNA. Thus, these data indicated that pre-treatment of quinones could resist the deterioration of mitochondrial function caused by doxorubicin, via regulating nucleoid morphology and replication activity.

In order to further solidify the case of using ubiquinone as a potential treatment approach by emphasizing its effect on nucleoid morphology in the context of mitochondrial disorders, I used two ubiquinone analogues with improved bioavailability – idebenone and vatiquinone^{55,56}. Similar experiments with pre-treatment with these quinones also showed their ability of resisting the nucleoid enlargement due to doxorubicin exposure, although the effect was slightly weaker than their natural counterparts such as menaquinone-4 (Fig 12F).

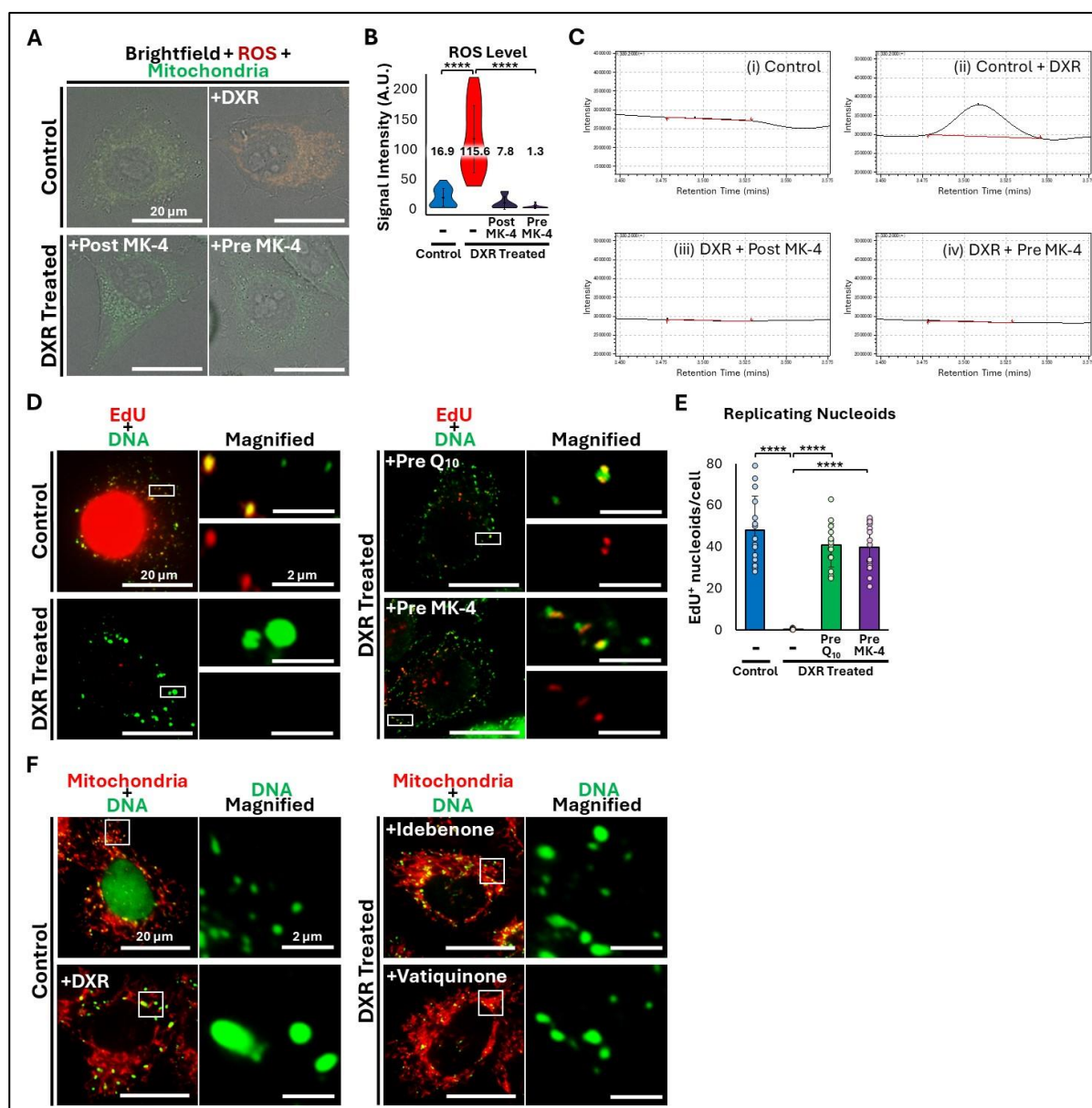


Fig 12. Outcome of pre-treatment with medically approved quinones on doxorubicin treated cells. A) Brightfield merged images from Fig 4G showing the cell boundary under 60x magnification (scale bar 20 μ m, magnified scale bar 2 μ m), B) quantification of ROS signal intensities (n=about 20 cells) and C) representative graphs showing the presence or absence of 2-Hydroxyethidium ions detected at m/z 330.2 in i) control, ii) control + DXR, iii) DXR + post MK-4 and iv) DXR + pre MK-4 cell sets. The red lines indicate the baseline. D) DXR treated HeLa cells subjected to pre-treatment of Q₁₀ or MK-4 followed by fluorescent microscopy after EdU labelling (replicating DNA/EdU, red; nucleoids, green) under 60x magnification (scale bar 20 μ m, magnified scale bar 2 μ m) and E) quantification replicating nucleoids (n=15 cells) in comparison to control cells. F) DXR treated HeLa cells subjected to pre-treatment with idebenone or vatiquinone followed by fluorescence microscopy (mitochondria, red; nucleoids, green) under 60x magnification (scale bar 20 μ m, magnified scale bar 2 μ m) in comparison to control cells. Error bars are shown in standard deviation values. **** $P < 0.0001$; where P values were determined by B,E) one-way ANOVA and Tukey-Kramer tests. B) Decimal numbers indicate the mean values of different populations.

Preventing cardiotoxicity of doxorubicin by maintaining nucleoids via pre-treatment of excess natural quinones

To further utilize this quinone-based nucleoid and respiratory regulation *in vivo*, I focused on the cardiotoxicity of anticancer drug doxorubicin in mice, since doxorubicin is known to have a potent cardiotoxic effects⁵⁷. When juvenile C57BL/6J male mice were subjected to doxorubicin exposure (Fig 13A), expression levels of two critical and specific cardiac failure biomarkers, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP)^{58,59,60,61} were both elevated in heart (Fig 13E,F); however these treatments caused no changes in whole heart weight (Fig 14E,F). Immunohistochemistry of the transverse sections of heart ventricles showed enlargement of nucleoids in the doxorubicin challenge group, compared to the vehicle group (Fig 13B,C; Fig 14D). Here I used menaquinone-4 and MitoTEMPO as pre-treatments before challenging the mice with doxorubicin (Fig 13A). As expected, the pre-treatment with both of these reagents quenched the reactive oxygen species in cardiac tissue of doxorubicin-treated mice as analysed by fluorescence microscopy (Fig 14A,B) and LC-MS analyses of 2-Hydroxyethidium levels (Fig 13D; Fig 14C). However, menaquinone-4, but not MitoTEMPO, was able to restore the nucleoid morphology (Fig 13B,C; Fig 14D), and showed a drastic reduction of the elevated ANP and BNP levels (Fig 13E,F). Although the cardiotoxic potency of doxorubicin has been long credited to superoxide formation⁶², quenching of superoxides by MitoTEMPO had weak or partial positive effect in rescuing heart failure (Fig 13D-F). Similarly, in the ubiquinone pre-treated group before doxorubicin treatment, the nucleoids remained small and dispersed, similar to the control heart (Fig 15A,B). The mRNA levels of ANP and BNP, both elevated in the doxorubicin challenge group, were significantly reduced in the ubiquinone pre-treatment set (Fig 15C,D). These data indicated that this unique function of quinones to support nucleoid morphology and function is critical for cardioprotection. Additional observation of the ventricular wall section showed a notable damage of myocardia under doxorubicin exposure which was rescued by pre-treatment with either of the quinones, but not by MitoTEMPO (Fig 13G; Fig 15E). The declination of total body weight, a hallmark of cardiac failure, was also seen in mice exposed to doxorubicin which was resisted in the menaquinone-4 pre-treated cohort and also partially in the MitoTEMPO pre-treated cohort as well (Fig 13H). Finally, I checked whether these quinone treatments affect the anticancer effect of doxorubicin. Although quinones prevent mitochondrial failure as the side effect of doxorubicin in heart, it does not interfere with the antiproliferative potency of the drug on HeLa cells (Fig 13I), similar to previous reports⁶³. Thus, I concluded here that quinone-based nucleoid structural maintenance prevents the deterioration of doxorubicin-induced cardiotoxicity specifically via maintaining nucleoid morphology (schematic model in Fig 13J).

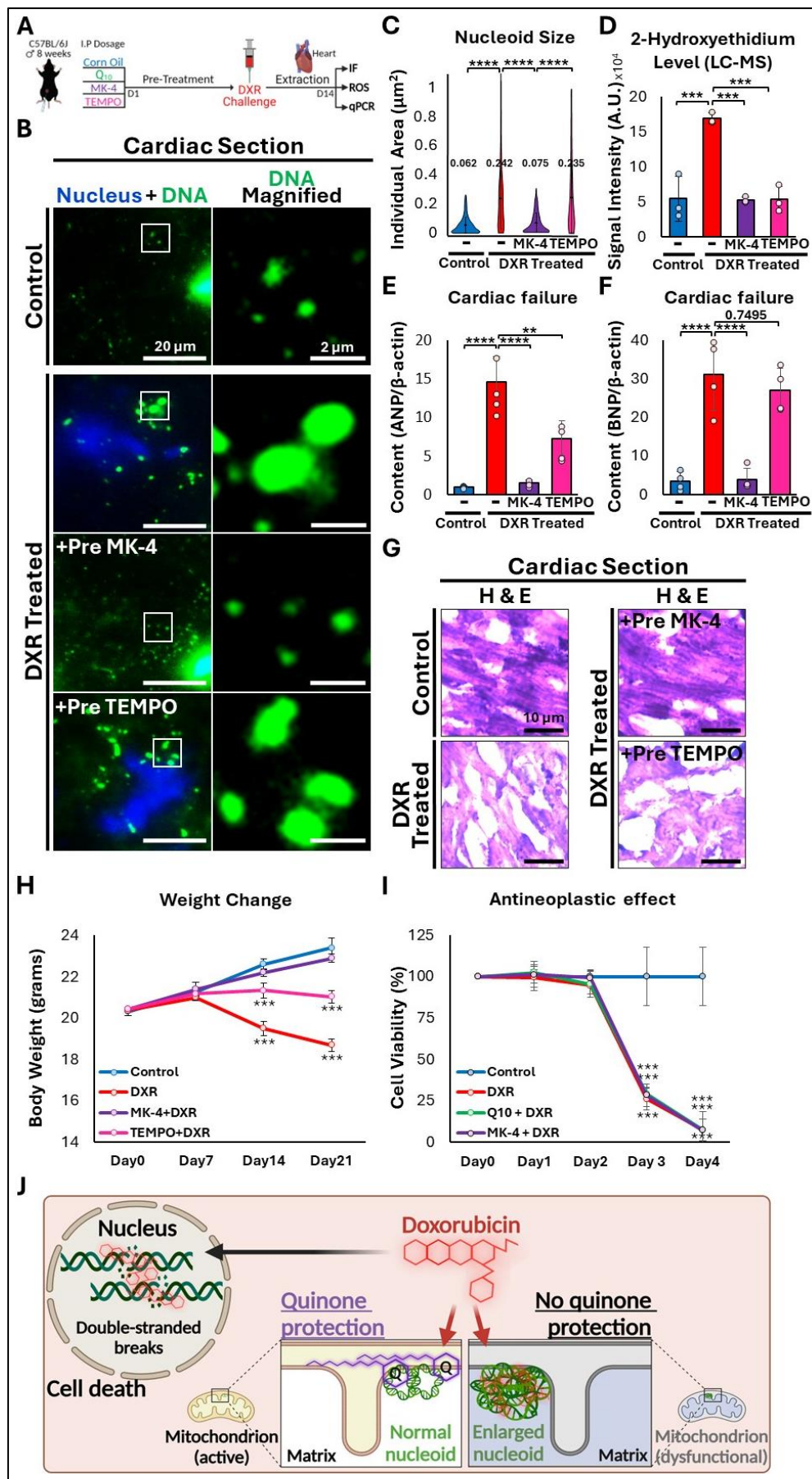


Fig 13. Quinone-based nucleoid regulation prevents aggravation of DXR induced cardiotoxicity *in vivo*.

A) Graphical representation of murine experimental model. Juvenile male mice were subjected to either MK-4 or TEMPO treatment prior to DXR exposure followed by B) fluorescence microscopy (nucleoids, green; nucleus, blue) of ventricular transverse sections under 60x magnification (scale bar 20 μ m, magnified scale bar 2 μ m), quantification of C) nucleoid sizes (n=more than 500 nucleoids), D) 2-Hydroxyethidium levels via LC-MS (n=3 independent tissue sets), E) Atrial Natriuretic Peptide (ANP) & F) Brain Natriuretic Peptide (BNP) levels by qPCR (n=4 mice), G) H&E staining of ventricular transverse sections under 60x magnification (scale bar 10 μ m) and H) profiling of body weights (n=3 mice) in comparison to control sets. I) Survival curve of cultured HeLa cells which were treated with DXR for 48 hrs, preceded by either Q₁₀, MK-4, or vehicle treatment for 24 hrs (n=3 independent cell sets). Each set value was counted as percentage survival calculation with respect to the control set. J) Graphical representation of doxorubicin action in mammalian cells. Error bars are shown in standard deviation values. **** P <0.0001, *** P <0.001 and ** P <0.01; where P values were determined by C-F,H,I) one-way ANOVA and Tukey-Kramer tests. C) Decimal numbers indicate the mean values of different populations.

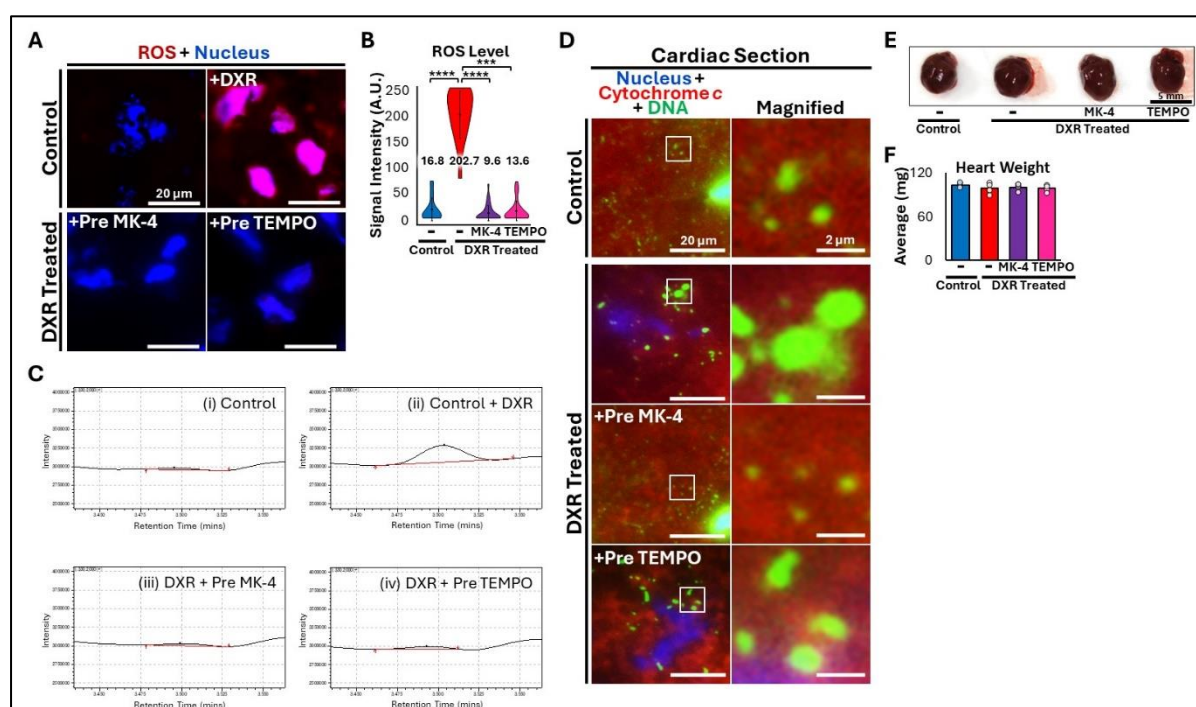


Fig 14. Measurement of reactive oxygen species and other characteristics in murine cardiac tissues. Juvenile male mice were subjected to either MK-4 or TEMPO treatment prior to DXR exposure followed by fluorescence microscopy of ventricular transverse sections A) (ROS, maroon) under 60x magnification (scale bar 20 μ m), and quantification of B) ROS signal intensities (n=about 40 DHE⁺ nuclei) in comparison to control sets. C) Representative graphs showing the presence or absence of 2-Hydroxyethidium ions detected at m/z 330.2 in i) control cardiac tissue, ii) control + DXR treated cardiac tissue, iii) DXR + Pre MK-4 treated cardiac tissue and iv) DXR + Pre TEMPO treated cardiac tissue sets. The red lines indicate the baseline. D) Mitochondrial marker cytochrome c (red) marked images (scale bar 20 μ m, magnified scale bar 2 μ m) from Fig 5B along with E) heart organ dimensions and F) weight (n=4 hearts) in comparison to control sets. Error bars are shown in standard deviation values. **** P <0.0001 and *** P <0.001; where P values were determined by B) one-way ANOVA and Tukey-Kramer tests. B) Decimal numbers indicate the mean values of different populations.

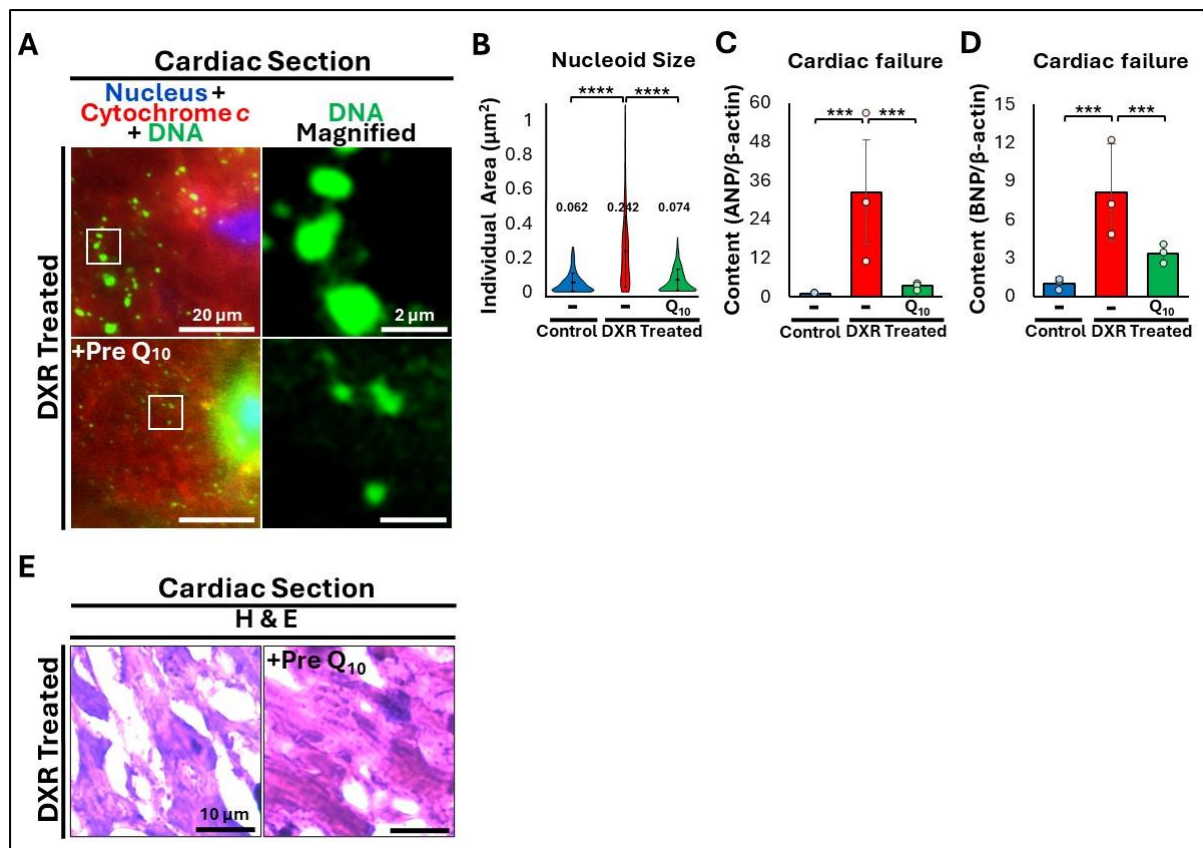


Fig 15. Result of Q₁₀ pre-treatment in juvenile male mouse which are exposed to doxorubicin. Mice subjected to the mentioned pre-treatment were subjected to A) fluorescence microscopy (nucleoids, green; nucleus, blue; cytochrome c, red) of ventricular transverse sections under 60x magnification (scale bar 20 μ m, magnified scale bar 2 μ m), quantification of B) nucleoid sizes (n=more than 500 nucleoids), C) Atrial Natriuretic Peptide (ANP) & D) Brain Natriuretic Peptide (BNP) levels by qPCR (n=3 mice), and E) H&E staining of ventricular transverse sections under 60x magnification (scale bar 10 μ m) in comparison to control sets. Error bars are shown in standard deviation values. **** P <0.0001 and *** P <0.001; where P values were determined by B-D) one-way ANOVA and Tukey-Kramer tests. B) Decimal numbers indicate the mean values of different populations.

Discussion

In mitochondrial diseases, issues with the mitochondrial genome has been widely attributed for mitochondrial respiratory dysfunction⁶⁴, and recent efforts are being made to create an mtDNA editing system to rescue mitochondrial failure⁶⁵. However, the dynamic nature of nucleoids and their potential involvement in disease progression has been overlooked. Nucleoid related abnormalities have been credited due to imbalance in fusion and fission of mitochondrial membrane^{29,30}; it was also recently showed that ATAD3 orchestrates nucleoid movement along the inner membrane²⁴. Here I showed that various potential pathological genes of mitochondrial diseases are engaged in altering nucleoid morphology, especially ADCK4 without affecting the mitochondrial length (Fig 3A,B). This also highlights the possible existence of a group of diseases that might arise due to defective mtDNA dynamics.

By maintaining nucleoid organisation, one can govern the integrity of the mitochondrial genome and resist the deterioration of cardiac function. In fact, nucleoid enlargement was reported in FBXL4 related mitochondrial encephalopathy³¹, and I, as well, have found many candidates that caused nucleoid enlargement. This set of candidate genes were initially identified via whole mtDNA and exome analyses from patients who showed childhood onset of mitochondrial respiratory chain complex deficiencies¹⁷. In addition to ADCK4, my RNAi screening showed that other cellular processes, some of them even being unrelated to mitochondria, can also cause nucleoid enlargement (graphical representation in Fig 1C). For example, genes related to metabolism and transfer of lipids, modulation of RNAs, such as tRNA charging, mRNA degradation and rRNA processing, can affect nucleoid structures. Cytoskeletal elements may also play a role in nucleoid structural maintenance, possibly via mediating cellular signals. I succeeded in observing nucleoid morphology in mice tissue sections (Fig 13B; Fig 14D; Fig 15A), nonetheless, the detection of nucleoid enlargement in clinical conditions remain a challenge. Further clinical advancements are needed to apply a detection system for enlarged nucleoids in patients to unravel the existence of other pathological conditions arising from nucleoid enlargement. In such cases, this quinone-based action of nucleoid structural maintenance could be applicable as a non-invasive mitochondrial DNA-targeted therapeutic method.

Cyclohexadienediones like ubiquinone has been credited with the functions of electron transportation and antioxidation within mitochondria⁶⁶. Its role in preventing ferroptosis has been recently reported, but it is again linked with its antioxidation potency to prevent cell death⁶⁷. My data shows clearly that ubiquinone can target and maintain nucleoids; a function that is not associated with its traditional roles. Maintaining nucleoids is not only inherent to ubiquinone, but to other natural quinones like menaquinone-4 and phyloquinone as well (Fig 6). In yeasts, studies have shown that loss of ubiquinone does affect the stability of the complexes belonging to the respiratory chain⁶⁸. However, I conclude that instead of affecting the stability of the proteins themselves, ubiquinone regulates morphology and function of nucleoids and rescues mtDNA copy number, which resists the loss of respiratory chain subunits (Fig

2). I also showed that ubiquinone directly mediates nucleoid formation on mitochondrial inner membrane; although I failed to show the direct interaction of nucleoids with inner membrane, since nucleoids in mitochondrial section were not observed by electron microscopy (Fig 3C), and fluorescent signals of lipids were not observed in my *in vitro* condition for an unknown reason (Fig 10A). As per these results, mtDNA localization on inner membrane should promote the subsequent transcription, translation, membrane insertion, and assembly processes of respiratory chain subunits (Fig 9). The loss of complex II factors in ADCK4-deficient cells could not be explained either by the function of ubiquinone or by the nucleoid enlargement since it is not coded by the mtDNA; the added ubiquinone also could not rescue the phenotype (Fig 2). Since re-introduction of ADCK4 rescued the reduction of complex II subunit as well as TFAM, it can be confirmed that the loss of complex II was a direct consequence of the absence of ADCK4 itself (Fig 2K), as reported previously⁴⁵, unrelated to ubiquinone synthesis. However, the domain essential for quinone synthesis was essential for the rescue of complex II reduction (Fig 4E). It was also unclear why the repression of other ubiquinone biosynthesis factors such as COQ3 and COQ7 did not affect complex II (Fig 5F). As a result, the underlying details of the role of ADCK4 in complex II remain to be clarified.

ADCK4 was reported to have a potential kinase motif, however, here I showed that it is not required for the regulation of nucleoid morphology. ADCK4 was also reported to attach to cardiolipin rich zones in the inner membrane by its KxGQ motif⁶⁹ to stabilize the ubiquinone biosynthetic complex, and here I showed that the KxGQ motif is essential for the nucleoids. It was also reported that proteins for ubiquinone biosynthesis form domains on mitochondrial membrane and that should mediate ER-mitochondria contact and lipid transfer in yeast and mammalian cells⁷⁰. I confirmed that COQ7, a member of ubiquinone biosynthetic complex, indeed had punctate distribution in mitochondria; however, the COQ7 puncta did not overlap with those of nucleoids (Fig 5A,B). In addition, ADCK4 itself showed a uniform distribution along mitochondria without forming any punctate domains (Fig 5C,D). Thus, these factors function in ubiquinone biosynthesis for maintaining nucleoid morphology and function, in a mitochondria-associated membrane (MAM)-independent manner.

Nucleoids are complexes made from mtDNA and proteins^{18,20,23}. Here I showed that ubiquinone supports nucleoid formation and its association to the mitochondrial inner membrane, given that cardiolipin is present sufficiently via *in vitro* experiments (Fig 9). With evidence of direct linking of DNA with membrane lipids via amphipathic agents⁷¹, as well as mtDNA being associated with specific lipid-rich zones within the inner membrane⁷², I considered the pairing of the amphipathic quinone molecule with a lipid for stabilizing the nucleoid structure (model in Fig 9F). The potential partner on membrane turned out to be cardiolipin and it has been previously reported that it is essential for enhancing the function of ubiquinone inside the inner membrane in flies⁷³. Additionally, the importance of cardiolipin biosynthesis in maintaining nucleoid structure had also previously identified⁷⁴. Natural quinones being amphipathic in

nature⁷⁵, therefore may need cardiolipin to stabilize themselves in the membrane with their hydrophobic tail. Thus, I conclude the existence of a quinone-based nucleoid formation on the inner membrane. But further analyses are needed to clarify the underlying mechanism on a molecular level. Nonetheless, I believe *in vivo*, both the protein components containing TFAM and membrane components containing quinone act in tandem with each other to maintain nucleoid integrity.

Widely used antineoplastic drug, doxorubicin, shows cardiotoxicity, although its mechanism is not completely clarified⁷⁶. It has previously been reported that the cardiotoxicity of doxorubicin can be attenuated by ubiquinone, via utilising its antioxidizing effect, and thus was considered essential for mitochondrial maintenance in the heart⁷⁷. However, my work shows that the antioxidizing effect alone cannot completely ameliorate the condition although it might have some protective effects (Fig 13). Although all cardiomyopathies may not occur due to nucleoid enlargement, it is still possible that this quinone-based nucleoid structural maintenance can prevent its aggravation and pathogenicity in a part of the disease. Doxorubicin has a highly planar structure which can intercalate with DNA molecules⁷⁸. I showed that biological quinones prevented enlargement of nucleoids, possibly by interacting with mtDNA via their planar, hydrophilic heads. This effect in nucleoid structural maintenance is only effective when the treatment with excess quinones is done prior to doxorubicin exposure and should prevent cardiac failure without reducing doxorubicin's antiproliferative potency (Figs 11 and 13). This timed regulation of nucleoids by exogenous quinones may explain why ubiquinone and its derivatives have shown relatively weak pharmacological effects in past clinical trials. They need to be administered in a time-dependent manner to obtain desirable outcomes.

Materials & Methods

Cell culture

HeLa cells and ADCK4 KO HeLa cells were grown in Dulbecco's Modified Eagle's Medium (Wako) supplemented with 10% fetal bovine serum (Invitrogen) under 37°C with a 5% CO₂ incubator.

RNAi screening and transfection

The data of Silencer Select RNAi oligonucleotides used for screening (a combination of 3 siRNAs for each target) and subsequent experiments are presented in Table 1. Only the negative control and TFAM siRNAs were from Nippongene. The siRNAs were introduced into HeLa cells by reverse and forward transfections using Lipofectamine RNAiMAX (Invitrogen) as per the manufacturer's protocol. Briefly, siRNAs, RNAiMAX, and Opti-MEM I (Gibco) were mixed in a 96-well plate or a glass-bottom dish, and then HeLa cells were seeded with Dulbecco's Modified Eagle's Medium (Wako) supplemented with 10% fetal bovine serum (Invitrogen). Fresh growth medium was added 24 hours post-transfection, and further transfections were performed for another 24 or 48 hours.

Live cell imaging

HeLa cells were cultured on glass-bottomed dishes or plastic bottom 96-well plates and stained with 10,000-fold diluted MitoTracker Red CMXRos (Invitrogen) for 10 + 5 minutes and 100,000-fold diluted SYBR Green I Nucleic Acid Gel Stain - 10,000X concentrate in DMSO (Invitrogen) for 5 minutes at 37°C. They were then washed twice with Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and changed to FluoroBrite DMEM (Gibco) containing 50 mM HEPES buffer at pH 7.4 (Gibco). After 30 min, the cells were observed under BZ-X710 Fluorescence Microscope (Keyence) or IX81 Microscope (Olympus).

To detect reactive oxygen species (ROS) or changes in mitochondrial membrane potential, MitoSOX Red Mitochondrial Superoxide Indicator (Thermo Fisher Scientific) or Tetramethylrhodamine methyl ester (TMRM) (Thermo Fisher Scientific) was dissolved in DMSO to make 5 mM or 100 μ M stock solutions, respectively, and they were further diluted to 1:1000 solution in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, which was then applied to the cells of interest, after washing the cells twice with PBS. After 10 minutes of incubation with the diluted MitoSOX staining solution, the cells were washed thrice with Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum solution before finally being put in FluoroBrite DMEM solution, followed by immediate observation under BZ-X710 Fluorescence Microscope.

Electron microscopy

HeLa cells were grown on culture dishes with a gridded polymer coverslip bottom (Ibidi). Then, they were fixed with 1% glutaraldehyde in 0.1 M cacodylate buffer at 4°C overnight. After washing five times with 0.1 M cacodylate buffer, the cells were further fixed with 1% osmium tetroxide solution at 4°C for 1 hour. They were washed five times with Milli-Q water, incubated in 2% uranyl acetate at 4°C for 1 hour, and washed five times with Milli-Q water. Samples were then dehydrated using an ethanol series (20, 50, 70, 90, and 99.5% for 5 minutes each and twice in 100% for 10 minutes), then followed by infiltration with Epon 812 (TAAB). Polymerization was performed at 60°C for 72 hours. To remove the polymer coverslip, the resin blocks in plastic dishes were immersed in toluene. Ultrathin sections (60-80 nm) were then cut with a diamond knife on an ultramicrotome Leica EM UC7 (Leica Microsystems) and placed on a piece of silicon wafer. The sections were stained with uranyl acetate followed by lead nitrate and observed with a field emission scanning electron microscope MERLIN VP Compact (Carl Zeiss) at 5 kV acceleration voltage using back scattered electron detector.

Immunoblots

10 µg of protein samples were made from cell lysates and then subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (using 10%, 13%, and 15% polyacrylamide gels), followed by their transfer to polyvinylidene fluoride membrane. A 5% skim milk solution in 1xTBS-Tween solution was used to block the membranes, followed by overnight incubation with primary antibodies. Then, the membranes were subjected to treatment with a mouse or rabbit horseradish peroxidase-conjugated secondary antibody before being detected using Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) and an ImageQuant LAS4000mini (GE Healthcare).

The list of antibodies used is mentioned in Table 2.

Real-time Polymerase Chain Reaction

QIAamp DNA Mini Kit (Qiagen) was used to extract DNA from cultured HeLa cells, following the manufacturer's instructions. KAPA SYBR FAST qPCR Master Mix(2X) ABI Prism Kit (Kapa Biosystems) was used for quantitative PCR with a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). Standard curves were produced by using 0.5, 1, 2, 4, and 8 ng of HeLa cell DNA for mtDNA amplification and 1, 2, 4, 8, and 16 ng of HeLa cell DNA for nuclear DNA amplification. The knockdown efficiency of ADCK4 in HeLa cells was also checked comparably with HPRT1 being used as internal control after extracting total RNA using TRIzol Reagent (Thermo Fisher Scientific) as per the manufacturer's protocol followed by subsequent

conversion to cDNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo) as per the manufacturer's protocol.

For the measurement of ANP and BNP levels, total RNA was extracted from murine hearts using TRIzol Reagent as per the manufacturer's protocol. The homogenization of the tissue section was done using a Micro Smash MS-100R (Tomy) at 3800 rpm for 30 seconds, twice. The subsequent conversion to cDNA was done using ReverTra Ace qPCR RT Master Mix with gDNA Remover, according to the manufacturer's protocol. Following this, quantification of natriuretic peptides was performed using a StepOnePlus Real-Time PCR System with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) according to the manufacturer's protocol. Normalization was done with β -actin.

The primers used for the amplification are as described in Table 3. Specifically, the primers used for amplifying mtDNA and nuclear DNA segments were as reported previously⁷⁹.

Oxygen Consumption Rate.

Oxygen consumption was measured at 37°C using an Agilent Seahorse XFe24 Analyzer (Agilent Technologies) as per the manufacturer's protocol. The cells were seeded on an XFe24 cell culture plate. On the following day, the cells were washed twice with fresh medium and then incubated in pre-warmed Agilent Seahorse XF Assay Media for Cell Mito Stress Test, supplemented with 10 mM glucose, 1 mM Pyruvate, 2 mM glutamine, and 1% fetal bovine serum (FBS) in a CO₂-free incubator at 37°C for 1 hour. Respiratory rate measurement was performed using 1 μ M Oligomycin, 1 μ M Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), and 0.5 μ M rotenone/Antimycin A. All parameters were determined as indicated in Seahorse XF Cell Mito Stress Test Report Generator, which was obtained from Wave Controller software. The results were normalized to a fixed amount of protein per well, and the protein concentration was quantified after each run by using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Immunocytochemistry

Cells grown on cover-slides were fixed for 10 minutes with 4% paraformaldehyde and then washed twice with PBS. They were then permeabilized for 5 minutes with 0.2% Triton X-100 in PBS solution. Following permeabilization and thorough PBS washing, the cells were then blocked for 1 hour with 5% skim milk in PBS solution and then subjected to primary antibody treatment for 1 hour at room temperature. This was followed by secondary antibody treatment, after which the sample was thoroughly washed with PBS and stained with Hoechst 33342 (Thermo Fisher Scientific) for 5

minutes. After that, the cover-slides were mounted on a glass slide using SlowFade Gold antifade reagent (Thermo Fisher Scientific) and fixed permanently.

The list of antibodies used is described in Table 4.

Super-resolution microscopy

For fixed and immunostained cells, super-resolution imaging was performed using the Nikon Super-Resolution Microscope System (N-SIM). The samples were observed under an Eclipse Ti microscope (Nikon) equipped with a CFI Apochromat TIRF 100XC immersion objective (numerical aperture 1.49) using 561 nm and 488 nm emission lasers. Obtained SIM images were reconstructed in NIS-Elements Advanced Research software (Nikon).

Replicating the mitochondrial nucleoid labelling assay

Cells grown on cover-slides, along with the necessary reagent treatments, were subjected to labelling with 15 μ M 5-ethynyl-2'-deoxyuridine or EdU (Invitrogen) for 8 hours. After incubation, the cells were thoroughly washed, fixed, permeabilized, and stained for EdU fluorescence using a Click-iT Plus EdU Imaging Kit (Invitrogen) as per the manufacturer's protocol. After that, the reaction mix was washed off from the cells, and then they were subjected to primary antibody treatment to label DNA for 1 hour at room temperature. This was followed by a 30-min treatment with secondary antibody, after which the sample was thoroughly washed with PBS. After that, the cover-slides were mounted on a glass slide using SlowFade Diamond antifade reagent (Thermo Fisher Scientific) and fixed permanently. The number of nucleoids containing EdU was counted using ImageJ2 software (v2.9.0).

The antibodies used are described in Table 4.

Immunohistochemistry

For staining of mitochondria and nucleoids in murine cardiac muscle samples, the entire heart was extracted, and the excess blood was wiped off. Then, the samples were embedded into Tissue-Tek O.C.T. Compound (Sakura Finetek) and frozen in liquid nitrogen. These frozen samples were then subjected to sectioning, with a 5 μ m thickness, using a CM3050S cryostat (Leica Biosystems). Next, the cardiac muscle sections were subjected to fixation using 4% paraformaldehyde solution for 20 minutes at room temperature. Following this, the samples were blocked using 2% bovine serum albumin solution in PBS for 30 minutes at room temperature. After that, the samples were incubated with primary antibodies, followed by secondary antibodies for 1 hour each at room temperature. Finally, the sections were stained with the Vector TrueVIEW Autofluorescence Quenching Kit (Vector Laboratories) and mounted on

glass slides using VECTASHIELD Vibrance Antifade Mounting Medium with DAPI (Vector Laboratories) as per the manufacturer's protocol. Before observations, the samples were left undisturbed for 2 hours at room temperature, under shade.

The list of antibodies used is mentioned in Table 5.

For hematoxylin and eosin (H&E) staining of murine heart tissue, after fixation, cardiac tissue sections were prepared as described above. Then, the excess OCT compound was thoroughly washed off with distilled water and stained with a Hematoxylin and Eosin Stain Kit (Vector Laboratories) as per the manufacturer's protocol. Immediately after staining, the tissue sections were examined.

To detect ROS in murine heart, after preparing cardiac tissue sections as mentioned above, the excess OCT compound was washed off thoroughly with PBS. Dihydroethidium (Wako) diluted in FluoroBrite DMEM to a final concentration of 2 μ M was then applied to the washed tissue sections, followed by incubation at 37°C for 30 minutes under shade. Finally, the sections were mounted on glass slides with VECTASHIELD Vibrance Antifade Mounting Medium with DAPI as per the manufacturer's protocol.

Reagent Treatment

Coenzyme Q₁₀ (Tokyo Chemical Industries), menaquinone-4 (Tokyo Chemical Industries), idebenone (Tokyo Chemical Industries), vatiquinone (Cayman Chemical), and phyloquinone (Wako) were dissolved in 99.5% ethanol to make a 10 mM stock solution. A 10 mM solution was applied to cultured cells, resulting in a final concentration of 100 μ M, and was left for treatment up to 48 hours. MitoTEMPO hydrate (Cayman Chemical) was dissolved in DMSO to a concentration of 100 μ M and was left for treatment up to 24 hours. Doxorubicin treatment was first dissolved in DMSO to make a 10 mM stock solution. This 10 mM solution was applied to cultured cells, resulting in a final concentration of 500 nM, and the cells were treated for 24 hours, either with or without a pre-treatment of 100 μ M of CoQ₁₀ or MK-4 for 24 hours.

Knockout cell creation

HeLa cells lacking ADCK4 were generated using the CRISPR/Cas9 gene editing system. For brief targeting of DNA sections specific to human ADCK4, two sets of oligomers serving as guide RNA were synthesized and cloned into pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmids, a gift from Feng Zhang separately (Addgene Plasmid #42230; <http://n2t.net/addgene:42230>; RRID:Addgene_42230) (55) and transfected into cells together. The oligomers were synthesized based on target recommendations from the CHOPCHOP database for human COQ8B, also known as ADCK4. After transfecting the cells with 1 μ g each of the synthesized plasmids for two consecutive days along with 0.2 μ g of pcDNA3.1(+) vector containing puromycin

resistant gene, the transfected cells were subjected to puromycin (4 µg/ml) selection for 48 hours. Following the selection single cells were sorted into 96-well plates. Surviving clones were picked, expanded, and further selected based on PCR results indicating insertions or deletions. The mutations were confirmed by genomic sequencing. The SDHD-lacking HeLa cell line generation was done as reported previously⁸⁰.

The list of oligomers used is mentioned in Table 6.

ADCK4 full-length and truncated constructs

ADCK4 cDNA was obtained with PCR using total RNA isolated from HeLa cells. The PCR product was cloned into the EcoRI and NotI sites of the pcDNA3.1(+) vector, which contains an HA epitope at the C-terminal end, and sequenced to rule out mutations introduced by PCR. All truncation mutants of ADCK4 were also cloned into the EcoRI and NotI sites of the pcDNA3.1(+) vector using standard molecular biology techniques with PCR. These PCR products were further sequenced to rule out off-target PCR-related mutations that may have been generated during the process. The PCR reactions were all done using the PrimeStar GXL Polymerase system (Takara).

Whenever rescue experiments were performed with these constructs, the cells of interest were transfected with a plasmid containing the construct (1 µg) or HA epitope-containing vector plasmid (1 µg) using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's protocol for 48 hours.

The list of primers used is described in Table 7.

Murine experimental design

All animal experiments were approved by The University of Osaka Graduate School of Science Animal Care and Use Committee (permission number: 2024-02-0), and was conducted according to The University of Osaka Animal Experimentation Regulations.

In the two-week experiment (n = 3,4 male C57BL/6J mice per group) to mimic acute DXR exposure, the mice (all 8 weeks old, obtained from CLEA Japan, Inc.) in the DXR challenge, CoQ protection + DXR challenge, MK-4 protection + DXR challenge, and MitoTEMPO protection + DXR challenge groups were injected with 12.5 mg/kg of DXR (diluted in equivalent amounts of DMSO and corn oil) on the 8th and 13th days, intraperitoneally. The control mice received corn oil. The mice in the CoQ protection + DXR challenge group received a continuous intraperitoneal injection of 50 mg/kg of CoQ, dissolved in corn oil. The mice in the MK-4 protection + DXR challenge group received a continual intraperitoneal injection of 50 mg/kg of MK-4. The mice in the MitoTEMPO protection + DXR challenge group received a continual intraperitoneal

injection of 0.1 mg/kg of MitoTEMPO for seven days before the first and for four days before the second round of DXR challenge. All groups of mice were sacrificed on the 14th day, and whole heart samples were extracted for tissue sectioning and RNA extraction. All murine experiments were approved by and conducted by the institutional guidelines of The University of Osaka.

For weight loss measurement, the aforementioned experimental groups except the CoQ protection group were subjected to the same experimental conditions, but instead of terminating the protocol on the 14th day, they were monitored for 21 days for changes in their body weights.

Extraction and LC-MS analysis of Coenzyme Q

Whole cell pellets containing 4×10^6 cells of ADCK4 KO and control HeLa cells were collected and snap frozen in liquid nitrogen. The frozen pellets were resuspended in 1 mL of ice-cold extraction buffer (200 mM ascorbate in 90% methanol), vortexed, and centrifuged at 15,000 rpm at 4°C for 5 minutes. The supernatants were collected and evaporated to dryness. The samples were then dissolved in an appropriate amount of the extraction buffer and analyzed using an LC-MS-8060 triple quadrupole mass spectrometer (Shimadzu). To detect the content of the oxidized form of CoQ (ubiquinone), reverse phase ion-pair chromatography was performed using a C18 column (100 x 2.1 mm, 1.7 μ m particle size; KINETEX) with isocratic flow of a solvent composed of 10 mM ammonium formate in methanol. The flow rate was set at 0.4 mL/min, and the column oven temperature was maintained at 40°C. The parameters for the heated electrospray ionization (ESI) in the positive ion mode under multiple reaction monitoring (MRM) were as follows: drying gas flow rate, 10 L/min; nebulizer gas flow rate, 3 L/min; heating gas flow rate, 10 L/min; interface temperature, 300°C; DL temperature, 250°C; heat block temperature, 400°C; CID gas, 270 kPa. The CoQ profiles were detected by targeted analysis of their ammonium adduct $[M+NH_4]^+$ through the following transition: $Q_{10} \ 881.4 \rightarrow 197.15 \ m/z$. Molecular species incorporating stable isotopes were identified by the $[M+6]$ transition for both the precursor and product.

Creation of 2-Hydroxyethidium standard for LC-MS analysis

A 100 μ M solution of dihydroethidium was prepared in anhydrous DMSO followed by its gradual addition to a 200 μ M solution of potassium superoxide (Strem Chemicals) in DMSO. The mixture was allowed to react for 30 minutes at room temperature with gentle shaking, while the reaction environment was free from atmospheric moisture. The reaction was then quenched by the addition of a small amount of acetic acid, followed by the removal of excess potassium superoxide by passing the mixture through a filter paper. The resulting product of 2-Hydroxyethidium was then purified and characterized by LC-MS analysis.

Extraction and LC-MS analysis of 2-Hydroxyethidium in cellular and tissue samples

Whole cell pellets containing 1×10^6 cells of ADCK4 KO, DXR-treated, and control HeLa cells were collected and snap frozen in liquid nitrogen after being stained with 5 μM dihydroethidium for 10 minutes at 37°C with or without the necessary Q10, MitoTEMPO, or MK-4 treatments. The frozen pellets were resuspended in 100 μL of ice-cold 80% methanol solution and subjected to ultrasonic disruption using a Bioruptor (set to High mode, 30 seconds on and 30 seconds off, for a total of 5 cycles). The sample was then centrifuged at $20,380 \times g$ for 5 minutes at 4°C , and the supernatant was collected. The supernatant was diluted fivefold with 0.1% formic acid in water before being analyzed by LC-MS. For tissue analysis, 50 mg of heart tissue from the control, DXR challenge, MK-4 protection + DXR, and MitoTEMPO protection + DXR challenge mice groups, as mentioned previously, were flash frozen in liquid nitrogen after being stained with 2 μM dihydroethidium for 30 minutes at 37°C . The frozen tissues were then homogenized in 200 μL of ice-cold 80% methanol solution using a BioMasher II, followed by centrifugation at $20,380 \times g$ for 5 minutes at 4°C . The supernatant was collected and diluted fivefold with 0.1% formic acid in water before LC-MS analysis. The samples were separated using an ACQUITY UPLC BEH C18 column (100 mm x 2.1 mm I.D., 1.7 μm particle size) and analyzed with an LCMS-8060 instrument (Shimadzu) in selected ion monitoring (SIM) mode for 2-OH-E⁺ positive ions at m/z 330.2⁸¹. The mobile phases consisted of 0.1% formic acid in water ("A") and 0.1% formic acid in acetonitrile ("B"). The gradient elution program was as follows: 0–1 min, 15% B; 1–5 min, 15–98% B; 5–7 min, 98% B; 7.1–10 min, 15% B. The flow rate was 0.3 mL/min, and the column oven temperature was maintained at 40°C . The ionization parameters were as follows: ionization mode, positive; nebulizer gas flow rate, 1.5 L/min; dry gas flow rate, 10 L/min; heating gas flow rate, 10 L/min; interface temperature, 400°C ; desolvation line (DL) temperature, 300°C ; and heat-block temperature, 500°C . Data processing was conducted using LabSolutions LCMS software (Shimadzu).

In vitro nucleoid reconstruction assay

His6- ΔN42 TFAM was expressed and purified by pET28 expression system in *Escherichia coli*. This recombinant protein or Fraction V Bovine Serum Albumin pH 7.0 (Wako) was incubated with circular plasmid DNA about 10 kbp in size in a 1000:1 ratio (i.e., 4000 ng of protein with 4 ng of plasmid) along with 0.2 M NaCl. The reaction mix was prepared to a volume of 10 μL using Tris-HCl based buffer and then incubated for 1 hour at 25°C using a thermal cycler. Post-incubation, SYBR Green I was added to the reaction mix at a 100,000-fold dilution and incubated at room temperature for 5 minutes in a dark enclosure, followed by observation using IX81 Microscope.

In vitro nucleoid-liposome complex formation assay

A circular plasmid DNA about 10 kbp in size was incubated with liposomes containing 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1,3-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol (CL(18:1)₄) in a 50:25:25 or 69:27:4 molar ratio (Avanti Polar Lipids) in a 1:3 ratio (i.e. 4 ng of plasmid with 12 ng of liposome). Along with 0.2 M NaCl, these reaction mixtures were further supplemented with or without 100 µM of CoQ and then made up to 10 µl using Tris-HCl based buffer, followed by 1 hour of incubation at 25°C using Biometra T3000 thermocycler. Post-incubation, SYBR Green I was added to the reaction mix at a 100,000-fold dilution and was incubated at room temperature for 5 minutes in a dark enclosure. Samples were then placed and sealed using a glass bar and cover glass slips, followed by observation using IX81 Microscope. The POPC-POPE-CL(18:1)₄ liposomes were first dissolved in a 5% solution of n-Octanoyl-N-methyl-D-glucamine (Dojindo) in Tris-HCl and glycerol-based buffer at pH 7.0 and then was subjected to dialysis to get the dissolved liposomes back without the detergent.

Quantification of fluorescence microscopy images

The mitochondria and nucleoid images, obtained using a Plan-Apochromat 60x/1.42 numerical aperture oil immersion objective attached to an Olympus iX81 Microscope, were first converted to grayscale images. Then, the images were tasked with 'Object Classification' where the classifier was manually trained to separate the mitochondrial network and nucleoid signals from the background noise and nuclear signals and then segmented using 'Simple Segmentation' of Ilastik toolkit (v.1.4.0). These segmented images were then subjected to 'Batch Processing' and then used for quantifying either the area of each nucleoid or the perimeter of mitochondrial network using ImageJ2 software (v2.9.0) via utilizing the 'Analyze Particles' tool. The intensity plot for illuminating the distribution of endogenous COQ7 and exogenous ADCK4 was done after the images were obtained using the same apparatus, followed by using the 'Plot Profile' tool of ImageJ2 software (v2.9.0).

The data for nucleoid quantification in cells, apart from the super-resolution microscopy, were taken from a single master set of data from one microscope and used accordingly across the figures when needed.

The quantification of ROS and TMRM intensities from the fluorescence microscopy images was done by first converting them to grayscale images, followed by manually selecting the mitochondrial signals using the 'Freehand ROI' tool and then subjecting them to mean gray value evaluation by using the 'Analyse' tool of ImageJ2 software (v2.9.0).

Cell viability assay

HeLa cells (1×10^5) were plated on 35 mm dishes. After 48 hours, cells were treated with 500 nM of doxorubicin for 96 hours. For CoQ and MK-4 treatment, cells were incubated with 100 μ M of both quinones, independently, for 24 hours before doxorubicin was added. On each of the four collection days, the cells were removed via trypsinization, and proliferation rates were measured using a CellDrop BF counter (DeNovix).

Statistical analyses and graphical representations

All the graphs were drawn using either Microsoft Excel or OriginPro. All statistical analyses for those graphs were done by using either OriginPro or Statistics Kingdom. The pictorial representations were done using BioRender and Microsoft PowerPoint. Chemical structures were drawn using Marvin JS.

Tables

Name	Sequence (5'-3')/Source
Negative Control Sense	UACUAUUCGACACGCGAAGtt
Negative Control Antisense	CUUCGCGUGUCGAAUAGUAtt
TFAM Sense	AAGAUUGAGAUGUGUUCACAAtt
TFAM Antisense	UUGUGAACACAUCUCAAUUCUtt
ADCK4#1	Thermo Fisher Scientific (s36677)
ADCK4#2	Thermo Fisher Scientific (s36676)
ADCK4#3	Thermo Fisher Scientific (s36678)
CRAT	Thermo Fisher Scientific (s3488)
CRAT	Thermo Fisher Scientific (s3487)
CRAT	Thermo Fisher Scientific (s3486)
DMD	Thermo Fisher Scientific (s4155)
DMD	Thermo Fisher Scientific (s4156)
DMD	Thermo Fisher Scientific (s4157)
DNAH5	Thermo Fisher Scientific (s4176)
DNAH5	Thermo Fisher Scientific (s4177)
DNAH5	Thermo Fisher Scientific (s4178)
GFM1	Thermo Fisher Scientific (s40061)
GFM1	Thermo Fisher Scientific (s40062)
GFM1	Thermo Fisher Scientific (s40060)
KARS	Thermo Fisher Scientific (s7675)
KARS	Thermo Fisher Scientific (s7675)
KARS	Thermo Fisher Scientific (s7675)
MAP1A	Thermo Fisher Scientific (s8496)
MAP1A	Thermo Fisher Scientific (s8498)
MAP1A	Thermo Fisher Scientific (s8497)
MPV17	Thermo Fisher Scientific (s226594)
MPV17	Thermo Fisher Scientific (s8952)
MPV17	Thermo Fisher Scientific (s8951)
MRPS23	Thermo Fisher Scientific (s28466)
MRPS23	Thermo Fisher Scientific (s28465)
MRPS23	Thermo Fisher Scientific (s28464)
NDUFAF6	Thermo Fisher Scientific (s44041)
NDUFAF6	Thermo Fisher Scientific (s44040)
NDUFAF6	Thermo Fisher Scientific (s44039)
QRSL1	Thermo Fisher Scientific (s30618)
QRSL1	Thermo Fisher Scientific (s30619)
QRSL1	Thermo Fisher Scientific (s30620)
RPS6KA6	Thermo Fisher Scientific (s26163)
RPS6KA6	Thermo Fisher Scientific (s26164)
RPS6KA6	Thermo Fisher Scientific (s26165)
SEC31B	Thermo Fisher Scientific (s24817)
SEC31B	Thermo Fisher Scientific (s24818)
SEC31B	Thermo Fisher Scientific (s24819)
STARD8	Thermo Fisher Scientific (s18825)
STARD8	Thermo Fisher Scientific (s18826)

STARD8	Thermo Fisher Scientific (s18827)
TTC37	Thermo Fisher Scientific (s18566)
TTC37	Thermo Fisher Scientific (s18567)
TTC37	Thermo Fisher Scientific (s18568)
WDR75	Thermo Fisher Scientific (s38530)
WDR75	Thermo Fisher Scientific (s38531)
WDR75	Thermo Fisher Scientific (s38532)

Table1. List of siRNAs used for screening and subsequent experiments.

Name	Source
Mouse Monoclonal Anti-OXPHOS Cocktail	Abcam (110413)
Mouse Polyclonal Anti-TFAM	Abnova (H00007019-B01P)
Rabbit Monoclonal Anti-GAPDH (D16H11)	Cell Signalling Technology (5174)
Rabbit Polyclonal Anti-TOMM40	Proteintech (18409-1-AP)
Mouse Monoclonal Anti-Drp1	BD Biosciences (611113)
Mouse Monoclonal Anti-Mfn2	Abnova (H00009927-M03)
Rabbit Polyclonal Anti-POLG2	Proteintech (10997-2-AP)
Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, HRP	Thermo Fisher Scientific (G-21040)
Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, HRP	Thermo Fisher Scientific (G-21234)

Table2. List of antibodies used for immunoblotting analyses.

Name	Sequence (5'-3')
ADCK4 Forward	CTAAGCCAGGACCTGCGGAAC
ADCK4 Reverse	GCAGACACAGCGTCAGGAGC
HPRT1 Forward	TGACACTGGCAAAACAATGCA
HPRT1 Reverse	GGTCCTTTTCACCAGCAAGCT
mtDNA Amp Forward	CTTCTGGCCACAGCACTTAAAC
mtDNA Amp Reverse	GCTGGTGTTAGGGTTCTTTGTTTT
nDNA Amp Forward	GCTGGGTAGCTCTTAAACAATGTATTCA
nDNA Amp Reverse	CCATGTACTAACAATGTCTAAATGGT
ANP Forward	GAGAAGATGCCGGTAGAAGA
ANP Reverse	AGAGGGCAGATCTATCGGA
BNP Forward	CTGCTGGAGCTGATAAGAGA
BNP Reverse	TGAGATATGTGTCACCTTGGA
β-Actin Forward	GGTCATCACTATTGGCAACGAG
β-Actin Reverse	GTCAGCAATGCCTGGGTACA

Table3. List of primers used for real-time polymerase chain reactions.

Name	Source
Mouse Monoclonal Anti-DNA	PROGEN (61014)
Rabbit Polyclonal Anti-HA.11	Covance (PRB-101C)
Rabbit Polyclonal Anti-TOMM40	Proteintech (18409-1-AP)
Mouse Monoclonal Anti-Tom20	Santa Cruz Biotechnology (sc-17764)
Rabbit Polyclonal Anti-COQ7	Proteintech (15083-1-AP)
Mouse Monoclonal Anti-Cytochrome c	Cell Signalling Technology (12963)

Goat anti-Rabbit IgG (H+L) Highly Cross Adsorbed Secondary Antibody, Alexa Fluor 568	Thermo Fisher Scientific (A-11036)
Goat anti-Mouse IgG2a (γ2a) Cross Adsorbed Secondary Antibody, Alexa Fluor 647	Thermo Fisher Scientific (A-21241)
Goat anti-Mouse IgM (Heavy chain) Cross Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific (A-21042)
Goat anti-Mouse IgG (H+L) Highly Cross Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific (A-11029)

Table4. List of antibodies used for immunocytochemistry of HeLa cells.

Name	Source
Mouse Monoclonal Anti-Cytochrome c	BD Pharmigen (556432)
Mouse Monoclonal Anti-DNA	PROGEN (61014)
Goat anti-Mouse IgM (Heavy chain) Cross Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific (A-21042)
Goat anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody, Alexa Fluor 568	Thermo Fisher Scientific (A-21124)

Table5. List of antibodies used for immunohistochemistry of murine heart.

Name	Sequence (5'-3')
gRNA Forward Site 1	CACCGGCACCTTGCGTTCTCGAGAG
gRNA Reverse Site 1	AAACCTCTCGAGAACGCAAGGTGCC
gRNA Forward Site 2	CACCGCTCGAGAGCGGTCACTCAGC
gRNA Reverse Site 2	AAACGCTGAGTGACCGCTCTCGAGC

Table6. List of oligomers used for gRNA synthesis in ADCK4 KO cell creation.

Name	Sequence (5'-3')
ΔKVGQ Forward	CGCCCTCATGCTCAGCATCCAGGAC
ΔKVGQ Reverse	CTGAGCATGAGGGCGGCCCTC
ΔAAASIGQV Forward	GTGCCCTTTCACCAGGGCCTGCTG
ΔAAASIGQV Reverse	CTGGTGAAAGGGCACCTCCTCCAAGGA
ADCK4-Full Forward	ATGTGGCTGAAGGTGGGGGGCCT
ADCK4 Full Reverse	TGAGGGATCCACCCAGGAGTCCCCT

Table7. List of oligomers used for ADCK4 full length and truncated constructs' creation.

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References

1. Nolfi-Donagan, Deirdre et al. Mitochondrial electron transport chain: Oxidative phosphorylation, oxidant production, and methods of measurement. *Redox biology* 37, 101674 (2020).
2. Sparagna GC, Gunter KK, Sheu SS, Gunter TE. Mitochondrial calcium uptake from physiological-type pulses of calcium. A description of the rapid uptake mode. *The Journal of biological chemistry* 270, 27510-27515 (1995).
3. Green DR, Reed JC. Mitochondria and apoptosis. *Science* 281, 1309-1312 (1998).
4. Harman D. Aging: a theory based on free radical and radiation chemistry. *J Gerontol* 11, 298-300 (1956).
5. Hanada Y, et al. MAVS is energized by Mff which senses mitochondrial metabolism via AMPK for acute antiviral immunity. *Nature communications* 11, 5711 (2020).
6. Gray, M W et al. Mitochondrial evolution. *Science* 283, 1476-81 (1999).
7. Martin, W et al. An overview of endosymbiotic models for the origins of eukaryotes, their ATP-producing organelles (mitochondria and hydrogenosomes), and their heterotrophic lifestyle. *Biological chemistry* 382, 1521-39 (2001).
8. Dyall, Sabrina D et al. Ancient invasions: from endosymbionts to organelles. *Science* 304, 253-7 (2004).
9. Ehlers, EH, Kölliker, A, & Seibold, KT Ernst von. *Zeitschrift für wissenschaftliche Zoologie* 8, 311-325 (1848).
10. Nass, M M, and S Nass. Intramitochondrial Fibers With DNA Characteristics. I. Fixation and electron staining reactions. *The Journal of cell biology* 19, 593-611 (1963).
11. Taanman JW. The mitochondrial genome: structure, transcription, translation and replication. *Biochimica et biophysica acta* 1410, 103-123 (1999).
12. Anderson S, et al. Sequence and organization of the human mitochondrial genome. *Nature* 290, 457-465 (1981).
13. Schaefer, Andrew M et al. The epidemiology of mitochondrial disorders--past, present and future. *Biochimica et biophysica acta* 1659, 115-20 (2004).
14. Wallace, Douglas C. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annual review of genetics* 39, 359-407 (2005).
15. El-Hattab, Ayman W, and Fernando Scaglia. Mitochondrial DNA depletion syndromes: review and updates of genetic basis, manifestations, and therapeutic

options. The journal of the American Society for Experimental NeuroTherapeutics 10, 186-98 (2013).

16. Taylor, RW, and Turnbull, DM. Mitochondrial DNA mutations in human disease. *Nature reviews. Genetics* 6, 389-402 (2005).

17. Kohda M, *et al.* A Comprehensive Genomic Analysis Reveals the Genetic Landscape of Mitochondrial Respiratory Chain Complex Deficiencies. *PLoS genetics* 12, e1005679 (2016).

18. Hensen F, Cansiz S, Gerhold JM, Spelbrink JN. To be or not to be a nucleoid protein: a comparison of mass-spectrometry based approaches in the identification of potential mtDNA-nucleoid associated proteins. *Biochimie* 100, 219-226 (2014).

19. Kang D, Kim SH, Hamasaki N. Mitochondrial transcription factor A (TFAM): roles in maintenance of mtDNA and cellular functions. *Mitochondrion* 7, 39-44 (2007).

20. Korhonen JA, Gaspari M, Falkenberg M. TWINKLE Has 5' -> 3' DNA helicase activity and is specifically stimulated by mitochondrial single-stranded DNA-binding protein. *The Journal of biological chemistry* 278, 48627-48632 (2003).

21. Kasashima K, Sumitani M, Endo H. Human mitochondrial transcription factor A is required for the segregation of mitochondrial DNA in cultured cells. *Experimental cell research* 317, 210-220 (2011).

22. Larsson NG, *et al.* Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nature genetics* 18, 231-236 (1998).

23. He J, *et al.* The AAA+ protein ATAD3 has displacement loop binding properties and is involved in mitochondrial nucleoid organization. *The Journal of cell biology* 176, 141-146 (2007).

24. Ishihara T, Ban-Ishihara R, Ota A, Ishihara N. Mitochondrial nucleoid trafficking regulated by the inner-membrane AAA-ATPase ATAD3A modulates respiratory complex formation. *Proceedings of the National Academy of Sciences of the United States of America* 119, e2210730119 (2022).

25. Brüser C, Keller-Findeisen J, Jakobs S. The TFAM-to-mtDNA ratio defines inner-cellular nucleoid populations with distinct activity levels. *Cell Rep* 37, 110000 (2021).

26. Iborra FJ, Kimura H, Cook PR. The functional organization of mitochondrial genomes in human cells. *BMC Biol* 2, 9 (2004).

27. Lewis SC, Uchiyama LF, Nunnari J. ER-mitochondria contacts couple mtDNA synthesis with mitochondrial division in human cells. *Science* 353, aaf5549 (2016).

28. Ota A, Ishihara T, Ishihara N. Mitochondrial nucleoid morphology and respiratory function are altered in Drp1-deficient HeLa cells. *Journal of biochemistry* 167, 287-294 (2020).
29. Ban-Ishihara R, Ishihara T, Sasaki N, Mihara K, Ishihara N. Dynamics of nucleoid structure regulated by mitochondrial fission contributes to cristae reformation and release of cytochrome c. *Proceedings of the National Academy of Sciences of the United States of America* 110, 11863-11868 (2013).
30. Ishihara T, *et al.* Dynamics of mitochondrial DNA nucleoids regulated by mitochondrial fission is essential for maintenance of homogeneously active mitochondria during neonatal heart development. *Molecular and cellular biology* 35, 211-223 (2015).
31. Bonnen PE, *et al.* Mutations in FBXL4 cause mitochondrial encephalopathy and a disorder of mitochondrial DNA maintenance. *Am J Hum Genet* 93, 471-481 (2013).
32. Crane FL, Hatefi Y, Lester RL, Widmer C. Isolation of a quinone from beef heart mitochondria. *Biochimica et biophysica acta* 25, 220-221 (1957).
33. Tan AK, Ramsay RR, Singer TP, Miyoshi H. Comparison of the structures of the quinone-binding sites in beef heart mitochondria. *The Journal of biological chemistry* 268, 19328-19333 (1993).
34. Szkopinska A. Ubiquinone. Biosynthesis of quinone ring and its isoprenoid side chain. Intracellular localization. *Acta Biochim Pol* 47, 469-480 (2000).
35. Olson, R E, and H Rudney. Biosynthesis of ubiquinone. *Vitamins and hormones* 40, 1-43 (1983).
36. Wang Y, Hekimi S. Molecular genetics of ubiquinone biosynthesis in animals. *Crit Rev Biochem Mol Biol* 48, 69-88 (2013).
37. Hargreaves IP. Coenzyme Q10 as a therapy for mitochondrial disease. *Int J Biochem Cell Biol* 49, 105-111 (2014).
38. Prole DL, Chinnery PF, Jones NS. Visualizing, quantifying, and manipulating mitochondrial DNA in vivo. *The Journal of biological chemistry* 295, 17588-17601 (2020).
39. Atmaca M, *et al.* Follow-up results of patients with ADCK4 mutations and the efficacy of CoQ10 treatment. *Pediatr Nephrol* 32, 1369-1375 (2017).
40. Korkmaz E, *et al.* ADCK4-Associated Glomerulopathy Causes Adolescence-Onset FSGS. *J Am Soc Nephrol* 27, 63-68 (2016).
41. Hughes BG, Harrison PM, Hekimi S. Estimating the occurrence of primary ubiquinone deficiency by analysis of large-scale sequencing data. *Sci Rep* 7, 17744 (2017).

42. Drovandi S, *et al.* Oral Coenzyme Q10 supplementation leads to better preservation of kidney function in steroid-resistant nephrotic syndrome due to primary Coenzyme Q10 deficiency. *Kidney Int* 102, 604-612 (2022).
43. Desbats MA, Lunardi G, Doimo M, Trevisson E, Salviati L. Genetic bases and clinical manifestations of coenzyme Q10 (CoQ 10) deficiency. *Journal of inherited metabolic disease* 38, 145-156 (2015).
44. Stefely JA, *et al.* Cerebellar Ataxia and Coenzyme Q Deficiency through Loss of Unorthodox Kinase Activity. *Molecular cell* 63, 608-620 (2016).
45. Widmeier E, *et al.* ADCK4 Deficiency Destabilizes the Coenzyme Q Complex, Which Is Rescued by 2,4-Dihydroxybenzoic Acid Treatment. *J Am Soc Nephrol* 31, 1191-1211 (2020).
46. Cerqua C, *et al.* Vitamin K2 cannot substitute Coenzyme Q(10) as electron carrier in the mitochondrial respiratory chain of mammalian cells. *Sci Rep* 9, 6553 (2019).
47. Murphy MP, *et al.* Guidelines for measuring reactive oxygen species and oxidative damage in cells and in vivo. *Nat Metab* 4, 651-662 (2022).
48. Kanki T, *et al.* Mitochondrial nucleoid and transcription factor A. *Annals of the New York Academy of Sciences* 1011, 61-68 (2004).
49. Ban T, *et al.* Molecular basis of selective mitochondrial fusion by heterotypic action between OPA1 and cardiolipin. *Nature cell biology* 19, 856-863 (2017).
50. Kukat C, Larsson NG. mtDNA makes a U-turn for the mitochondrial nucleoid. *Trends in cell biology* 23, 457-463 (2013).
51. Kozhukhar N, Alexeyev MF. 35 Years of TFAM Research: Old Protein, New Puzzles. *Biology (Basel)* 12, (2023).
52. Takemura G, Fujiwara H. Doxorubicin-induced cardiomyopathy from the cardiotoxic mechanisms to management. *Prog Cardiovasc Dis* 49, 330-352 (2007).
53. Ashley N, Poulton J. Anticancer DNA intercalators cause p53-dependent mitochondrial DNA nucleoid re-modelling. *Oncogene* 28, 3880-3891 (2009).
54. Lee SR, Han J. Mitochondrial Nucleoid: Shield and Switch of the Mitochondrial Genome. *Oxid Med Cell Longev* 2017, 8060949 (2017).
55. Suarez-Rivero JM, *et al.* Coenzyme Q(10) Analogues: Benefits and Challenges for Therapeutics. *Antioxidants (Basel)* 10, (2021).
56. Neergheen VC, A.; Wainwright, L.; Yubero, D.; Montero, R.; Artuch, R.; Hargreaves, I. Coenzyme Q10 in the Treatment of Mitochondrial Disease. *J Inborn Errors Metab Screen* 5, (2017).

57. Ichikawa Y, *et al.* Cardiotoxicity of doxorubicin is mediated through mitochondrial iron accumulation. *The Journal of clinical investigation* 124, 617-630 (2014).
58. Qi W, Boliang W, Xiaoxi T, Guoqiang F, Jianbo X, Gang W. Cardamonin protects against doxorubicin-induced cardiotoxicity in mice by restraining oxidative stress and inflammation associated with Nrf2 signaling. *Biomed Pharmacother* 122, 109547 (2020).
59. Tsutamoto T, *et al.* Attenuation of compensation of endogenous cardiac natriuretic peptide system in chronic heart failure: prognostic role of plasma brain natriuretic peptide concentration in patients with chronic symptomatic left ventricular dysfunction. *Circulation* 96, 509-516 (1997).
60. Nishikimi T, Maeda N, Matsuoka H. The role of natriuretic peptides in cardioprotection. *Cardiovascular research* 69, 318-328 (2006).
61. Sergeeva IA, Christoffels VM. Regulation of expression of atrial and brain natriuretic peptide, biomarkers for heart development and disease. *Biochimica et biophysica acta* 1832, 2403-2413 (2013).
62. Cappetta D, *et al.* Oxidative Stress and Cellular Response to Doxorubicin: A Common Factor in the Complex Milieu of Anthracycline Cardiotoxicity. *Oxid Med Cell Longev* 2017, 1521020 (2017).
63. Greenlee H, Shaw J, Lau YI, Naini A, Maurer M. Lack of effect of coenzyme q10 on doxorubicin cytotoxicity in breast cancer cell cultures. *Integr Cancer Ther* 11, 243-250 (2012).
64. Stenton SL, Prokisch H. Genetics of mitochondrial diseases: Identifying mutations to help diagnosis. *EBioMedicine* 56, 102784 (2020).
65. Mok BY, *et al.* A bacterial cytidine deaminase toxin enables CRISPR-free mitochondrial base editing. *Nature* 583, 631-637 (2020).
66. Maroz A, Anderson RF, Smith RA, Murphy MP. Reactivity of ubiquinone and ubiquinol with superoxide and the hydroperoxyl radical: implications for in vivo antioxidant activity. *Free Radic Biol Med* 46, 105-109 (2009).
67. Deshwal S, *et al.* Mitochondria regulate intracellular coenzyme Q transport and ferroptotic resistance via STARD7. *Nature cell biology* 25, 246-257 (2023).
68. Parey K, *et al.* Cryo-EM structure of respiratory complex I at work. *eLife* 7, (2018).
69. Reidenbach AG, *et al.* Conserved Lipid and Small-Molecule Modulation of COQ8 Reveals Regulation of the Ancient Kinase-like UbiB Family. *Cell Chem Biol* 25, 154-165 e111 (2018).

70. Subramanian K, *et al.* Coenzyme Q biosynthetic proteins assemble in a substrate-dependent manner into domains at ER-mitochondria contacts. *The Journal of cell biology* 218, 1353-1369 (2019).
71. Elbahnasawy MA, Nasr ML. DNA-nanostructure-templated assembly of planar and curved lipid-bilayer membranes. *Front Chem* 10, 1047874 (2022).
72. Gerhold JM, *et al.* Human Mitochondrial DNA-Protein Complexes Attach to a Cholesterol-Rich Membrane Structure. *Sci Rep* 5, 15292 (2015).
73. Vos M, *et al.* Cardiolipin promotes electron transport between ubiquinone and complex I to rescue PINK1 deficiency. *The Journal of cell biology* 216, 695-708 (2017).
74. Ban T, Kohno H, Ishihara T, Ishihara N. Relationship between OPA1 and cardiolipin in mitochondrial inner-membrane fusion. *Biochim Biophys Acta Bioenerg* 1859, 951-957 (2018).
75. Samoilova RI, Crofts AR, Dikanov SA. Reaction of superoxide radical with quinone molecules. *J Phys Chem A* 115, 11589-11593 (2011).
76. Maillet A, *et al.* Modeling Doxorubicin-Induced Cardiotoxicity in Human Pluripotent Stem Cell Derived-Cardiomyocytes. *Sci Rep* 6, 25333 (2016).
77. Conklin KA. Coenzyme q10 for prevention of anthracycline-induced cardiotoxicity. *Integr Cancer Ther* 4, 110-130 (2005).
78. Yang F, Teves SS, Kemp CJ, Henikoff S. Doxorubicin, DNA torsion, and chromatin dynamics. *Biochimica et biophysica acta* 1845, 84-89 (2014).
79. Malik AN, Shahni R, Rodriguez-de-Ledesma A, Laftah A, Cunningham P. Mitochondrial DNA as a non-invasive biomarker: accurate quantification using real time quantitative PCR without co-amplification of pseudogenes and dilution bias. *Biochemical and biophysical research communications* 412, 1-7 (2011).
80. Chen CL, *et al.* SDHAF2 facilitates mitochondrial respiration through stabilizing succinate dehydrogenase and cytochrome c oxidase assemblies. *Mitochondrion* 79, 101952 (2024).
81. Wang ZY, *et al.* Development and validation of a LC/MS-based method for the measurement of intracellular superoxide anion. *Anal Chim Acta* 999, 107-113 (2018).