



# Epigenetic modification of miR-663 controls mitochondria-to-nucleus retrograde signaling and tumor progression

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The normal cellular function requires communication between mitochondria and the nucleus, termed mitochondria-to-nucleus retrograde signaling. Disruption of this mechanism has been implicated in the development of cancers. Many proteins are known modulators of retrograde signaling, but whether microRNAs (miRNAs) are also involved is unknown. We conducted an miRNA microarray analysis using RNA from a parental cell line, a Rho<sup>0</sup> line lacking mitochondrial DNA (mtDNA) and a Rho<sup>0</sup> line with restored mtDNA. We found that miR-663 was down-regulated in the mtDNA-depleted Rho<sup>0</sup> line. mtDNA restoration reversed this miRNA to parental level, suggesting that miR-663 may be epigenetically regulated by retrograde signaling. By using methylation-specific PCR and bisulfite sequencing we demonstrate that miR-663 promoter is epigenetically regulated not only by genetic but also by pharmacological disruption of oxidative phosphorylation (OXPHOS). Restoration of OXPHOS Complex I inhibitor-induced miR-663 expression by *N*-acetylcysteine suggested that reactive oxygen species (ROS) play a key role in epigenetic regulation of miR-663. We determined that miR-663 regulates the expression of nuclear-encoded respiratory chain subunits involved in Complexes I, II, III, and IV. miR-663 also controlled the expression of the Complexes I (NDUFAF1), II (SDHAF2), III (UQCC2), and IV (SCO1) assembly factors and was required for stability of respiratory supercomplexes. Furthermore, using luciferase assays, we found that miR-663 directly regulates UQCC2. The anti-miR-663 reduced OXPHOS complex activity and increased *in vitro* cellular proliferation and promoted tumor development *in vivo* in mice. We also found that increased miR-663 expression in breast tumors consistently correlates with increased patient survival. We provide the first evidence for miRNA controlling retrograde signaling, demonstrating its epigenetic regulation and its role in breast tumorigenesis.

Mitochondria are principally known for their role in producing cellular energy, although they are integral in other processes such as cellular growth, death, and signaling. The mitochondrial proteome is nuclear encoded except for 13 proteins encoded by the mitochondrial genome. Therefore, the mitochondrion must import over 99% of its proteome. As a result, the coordinated communication of the nuclear and mitochondrial genomes is required for numerous cellular processes. The cell accomplishes this coordination through the anterograde and retrograde dispatch of signals in the nucleus-to-mitochondria and mitochondria-to-nucleus directions, respectively. Although anterograde communication has received much focus in the past given the nucleus' major role in regulating mitochondrial structure and function, retrograde communication has become an increasingly important area of research. In mammals, it has been linked to metabolic stress response by activation of AMP-activated protein kinase (AMPK) and subsequent stimulation of PGC1- $\alpha$  to promote mitochondrial biogenesis; it has also been shown to intersect with calcium signaling via several calcium-regulated kinases such as protein kinase C and p38 MAPK (1). The pathway has also been shown to regulate the p53 tumor suppressor pathway (2, 3). Importantly, dozens of genes from several functional categories including transcription, cell signaling, and cell architecture are known to be involved in retrograde communication in breast cancer (2, 4, 5). Moreover, mitochondrial dysfunction is a hallmark of many cancers, suggesting an important role for retrograde signaling as well (6–9). Cytoplasmic hybrid (cybrid) studies, in which nuclear backgrounds are kept constant but mitochondrial genetic profiles are modified, suggest that retrograde signaling may play an important role in tumorigenesis (10, 11).

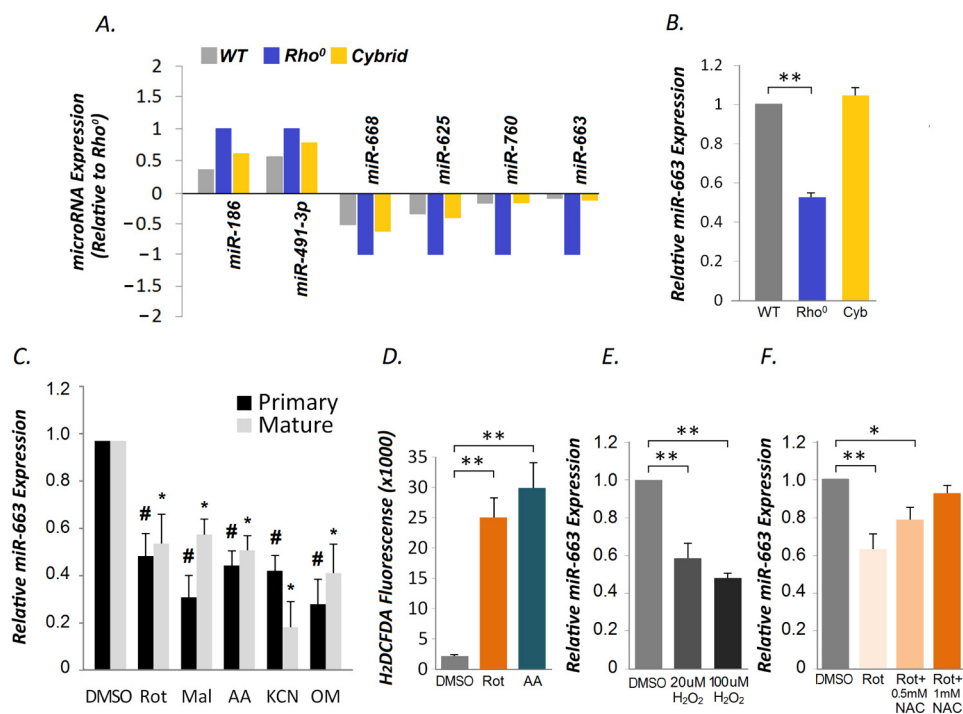
microRNAs (miRNAs)<sup>2</sup> are small, noncoding RNAs that impose genetic regulation by pairing with complementary sequences in a transcript's 3'-untranslated region (UTR). As a class of molecules, miRNAs are well-known to play important roles in a variety of cancers (12). The prospect of their utility as biomarkers for cancer is an increasingly important area of research (13, 14).

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This article contains supplemental Table S1 and supplemental Figs. S1–S4.

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<sup>2</sup> The abbreviations used are: miRNAs, microRNAs; UTR, untranslated region; ROS, reactive oxygen species; mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation; MSP, methylation-specific PCR; CM-H<sub>2</sub>DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester; TCGA, The Cancer Genome Atlas; BN-PAGE, Blue Native-polyacrylamide gel electrophoresis; CRISPR, clustered regularly interspaced short palindromic repeats; HMA, heteroduplex mobility assay; COBRA, combined bisulfite restriction analysis.



**Figure 1. miR-663 controls mitochondria-to-nucleus retrograde signaling.** *A*, results of a microRNA microarray using RNA from 143B parental (WT) cells, parental cells devoid of mtDNA (*Rho*<sup>0</sup>), and *Rho*<sup>0</sup> cells with restored mtDNA (*Cybrid*). The graph represents -fold change of the six most modulated microRNAs in the parental and cybrid lines relative to the *Rho*<sup>0</sup> line. *B*, confirmation of the microarray results showing down-regulation of miR-663. Real-time PCR confirmation of down-regulated miR-663 at the mature transcript level in *Rho*<sup>0</sup> cells compared with WT (parental) and cybrid cells. *C*, real-time PCR of the primary transcript and the mature miR-663 molecule shows down-regulation of both forms after inducing mitochondrial dysfunction at each complex in the electron transport chain. 143B cells were treated for 12 h with DMSO or inhibitors of mitochondrial oxidative phosphorylation (OXPHOS) complexes. *Rot* = rotenone (Complex I, 100 nM), *mal* = malonate (Complex II, 10 mM), *AA* = antimycin A (Complex III, 20  $\mu$ M), *KCN* = potassium cyanide (Complex IV, 1 mM), and *OM* = oligomycin (Complex V, 12.5  $\mu$ M). # denotes  $p < 0.01$  comparing primary transcript between DMSO and treatments. *D*, assessment of reactive oxygen species by detection of oxidized CM-H<sub>2</sub>DCFDA in 143B cells after treatment with rotenone or antimycin A. *E*, expression of primary miR-663 expression in 143B cells treated with H<sub>2</sub>O<sub>2</sub>. *F*, expression of primary miR-663 in 143B cells treated with rotenone alone or rotenone in combination with *N*-acetylcysteine. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  relative to DMSO. All real-time PCR data represent the average of three biological replicates. Error bars represent S.D.

miRNAs have been shown to regulate anterograde communication between the nucleus and mitochondria. For example, mitochondrial COX1 mRNA expression is increased by nuclear-encoded miR-181c (15), and miR-1 increases mitochondrial translation during muscle differentiation (16). Moreover, miR-214 modulates mitochondrial morphology by down-regulating MFN2 (17), and miR-7 regulates the permeability transition pore by targeting VDAC1 (18). It is clear that miRNAs play a role in mediating anterograde communication, but there are currently no reports of the involvement of miRNAs in retrograde communication. We show that the miR-663 is epigenetically regulated and suppresses tumor development. It regulates mitochondrial function and mediates the retrograde response.

## Results

### miR-663 mediates mitochondria-to-nucleus retrograde signaling and is regulated by reactive oxygen species (ROS)

The mitochondria-to-nucleus retrograde response is a cascade of molecular events of which our understanding is incomplete (5, 19, 20). To identify miRNAs in this pathway, we conducted an miRNA microarray using RNA from a parental cell line, its *Rho*<sup>0</sup> derivative devoid of mitochondrial DNA (mtDNA), and a cybrid line with repleted mtDNA from healthy platelets (Fig. 1A). The most significantly increased miRNAs in the *Rho*<sup>0</sup> line were hsa-miR-186 and hsa-491-3p; the most sig-

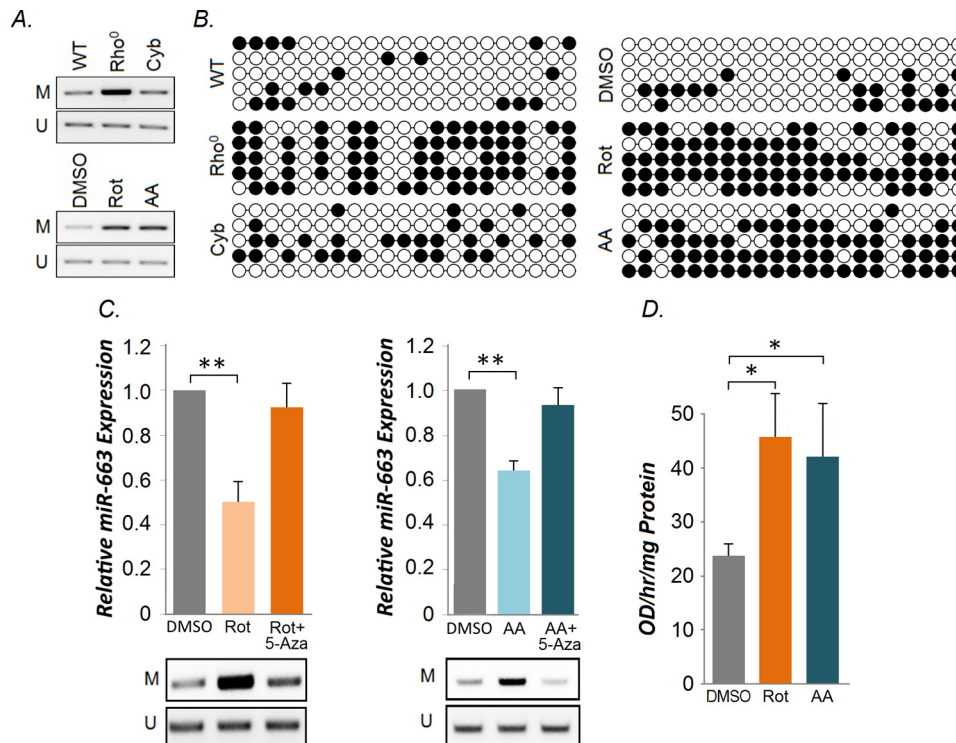
nificantly decreased miRNAs were hsa-miR-668, hsa-miR-625, hsa-miR-760, and hsa-miR-663. Restoration of mtDNA in cybrid cells restored expression of these miRNAs.

Because its role in breast cancer is not fully characterized, we selected miR-663 for validation by real-time PCR. Mature miR-663 expression was reduced by depletion of mtDNA, and repletion restored its expression to parental levels (Fig. 1B). Because the miR-663 expression is dependent on mitochondrial function, we hypothesized that ROS produced by the impairment of oxidative phosphorylation (OXPHOS) may be the regulatory signal (21, 22). We treated parental cells with OXPHOS complex inhibitors and found down-regulation of primary and mature miR-663 (Fig. 1C). Detection of oxidized CM-H<sub>2</sub>DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester) by FACS showed that mitochondrial impairment significantly increases reactive oxygen species (Fig. 1D). H<sub>2</sub>O<sub>2</sub> reduced primary miR-663 expression (Fig. 1E). Similarly, rotenone was able to down-regulate primary miR-663 expression, and the antioxidant *N*-acetylcysteine restored its expression (Fig. 1F). We conclude that mitochondria and mitochondrial ROS regulate miR-663.

### Mitochondria regulate miR-663 promoter methylation

We showed previously that loss of mtDNA reversibly alters DNA methylation (19), suggesting mitochondrial control over

## Retrograde miR-663 suppresses tumorigenesis



**Figure 2. Mitochondrial dysfunction induces hypermethylation of miR-663 promoter.** *A*, methylation-specific PCR assessing methylation of the miR-663 promoter in Rho<sup>0</sup> cells compared with WT or Cybrid cells and after treatment of WT cells with rotenone or antimycin A. *M* denotes methylated DNA amplified by primers specific for methylated DNA. *U* denotes unmethylated DNA amplified by primers specific for unmethylated DNA. *B*, bisulfite sequencing depicts 21 CpG dinucleotides present in the miR-663 promoter. Each row represents the sequencing results of a single clone. Each *black circle* represents a methylated CpG dinucleotide, and each *white circle* represents an unmethylated CpG dinucleotide. *C*, the *bar graphs* on top show real-time PCR of primary miR-663 in 143B cells after treatment with rotenone or antimycin A in the presence or absence of 5-aza-2'-deoxycytidine. The *lower panels* show MSP with rotenone or antimycin A in the presence or absence of 5-aza-2'-deoxycytidine. *D*, cellular methyltransferase activity after inhibition of Complex I (rotenone) or III (antimycin). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ . Error bars represent S.D. Real-time PCR data represent the average of three biological replicates. MSP and bisulfite sequencing results are representative of two biological replicates. Methyltransferase activity data represent two biological replicates run in triplicate.

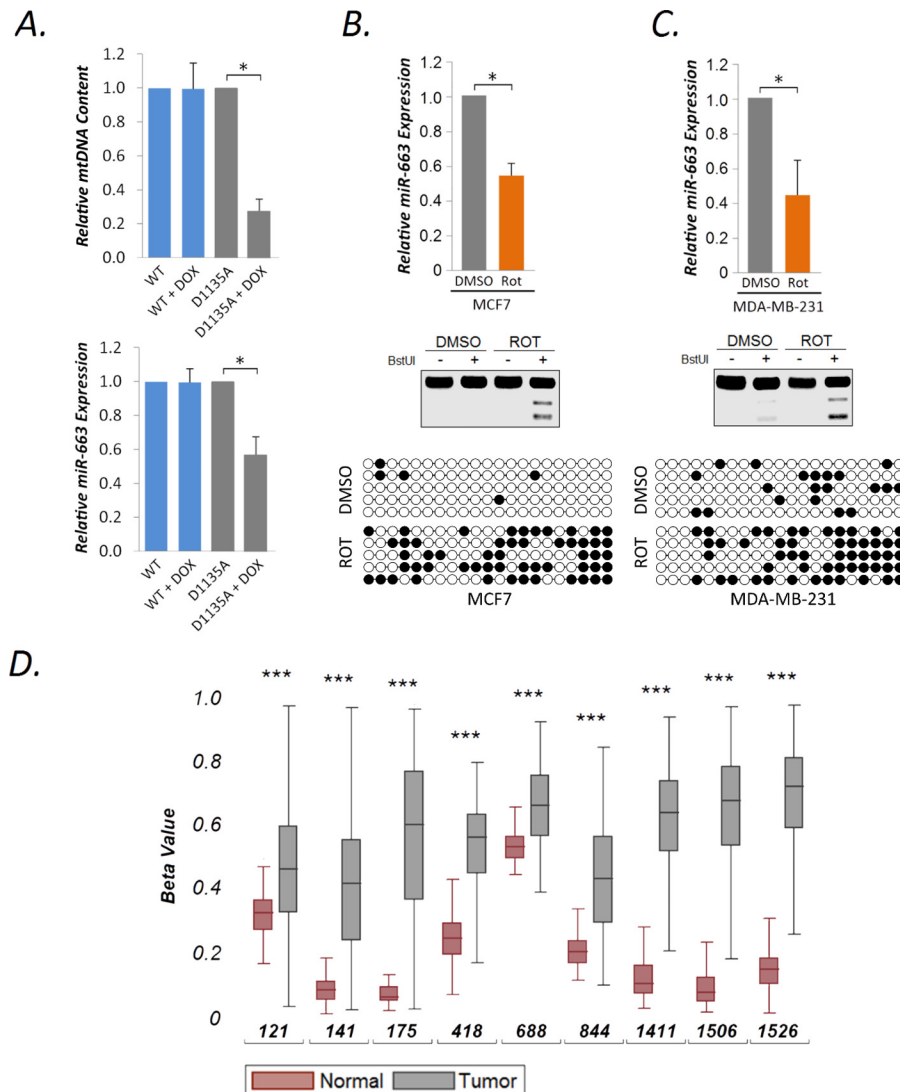
nuclear gene expression. Our current study demonstrates the regulation of miR-663 expression by mitochondrial function, and its reversibility suggests an epigenetic mechanism. We used methylation-specific PCR (MSP) and genomic bisulfite sequencing (GBS) to show that mitochondrial dysfunction deregulates miR-663 through DNA methylation. The miR-663 promoter was hypermethylated in Rho<sup>0</sup> (parental cells without mtDNA) cells compared with parental and cybrid cells, and the same was true of parental cells treated with rotenone or antimycin A (Fig. 2*A*). We confirmed this with genomic bisulfite sequencing (Fig. 2*B*). Furthermore, we found that hypermethylation and reduced expression of miR-663 by rotenone and antimycin A did not occur in the presence of 5-aza-2'-deoxycytidine, an inhibitor of DNA methylation (Fig. 2*C*). Importantly, treatment of cells with rotenone or antimycin A increased their methyltransferase activity (Fig. 2*D*).

We extended our study to breast cancer cells demonstrating that mitochondrial dysfunction, induced by depletion of mtDNA by a POLG-dominant negative mutant or chemically by rotenone, results in the down-regulation of miR-663 (Fig. 3, *A–C*). We also observed increased methylation of the miR-663 promoter after treatment with rotenone as shown by combined bisulfite restriction analysis (COBRA) and bisulfite sequencing (Fig. 3, *B* and *C*). We analyzed the methylation status of miR-663 in primary breast tumors from The Cancer Genome Atlas (TCGA) database. Consistent with our studies, we discovered

hypermethylation of the miR-663 promoter in tumors compared with normal samples (Fig. 3*D*). These data suggest that miR-663 is down-regulated by mitochondrial dysfunction through an epigenetic mechanism involving DNA hypermethylation and that miR-663 may play a role in cancer.

### miR-663 regulates nuclear-encoded OXPHOS subunits

Because miR-663 responds to mitochondrial dysfunction, we hypothesized a role for the miRNA in regulating mitochondrial function. To investigate the role of miR-663 in regulating the expression of nuclear-encoded mitochondrial genes, we transfected a control vector, an miR-663 expression vector or an anti-miR-663-expressing vector in MCF7 and MDA-MB-231 breast cancer cells. We achieved 3- to 3.5-fold up-regulation of miR-663 as assessed by real-time PCR (supplemental Fig. S2). Because the anti-miR-663 only sequesters miR-663 rather than degrading it, we determined miR-663 inhibition by analyzing the expression of known target genes. Significant down-regulation of miR-663 (supplemental Fig. 2) was demonstrated by increased expression of the well-established target genes, TGF- $\beta$ 1 (23–26) and JunB (27, 28). Western blotting of mitochondrial extracts with an antibody mixture against OXPHOS subunits for each complex showed that the anti-miR-663 reduced the expression of subunits in both MCF7 and MDA-MB-231 cells (Fig. 4*A*). We expanded our analysis to assess OXPHOS gene expression in MCF7 cells and found that mod-



**Figure 3. miR-663 is regulated by mitochondrial dysfunction and DNA methylation.** *A*, top panel shows quantification of mtDNA content after depletion of mtDNA in MCF7 cells. Depletion was achieved by activating a doxycycline-inducible dominant negative POLG mutant, D1135A. Bottom panel shows real-time PCR of the primary miR-663 transcript in MCF7 cells showing down-regulation of miR-663 after depletion of mtDNA. *B* and *C*, MCF7 cells (*B*) and MDA-MB-231 (*C*) treated with rotenone also had reduced expression of miR-663. Middle panels show COBRA analyses in which bisulfite converted DNA was digested with BstUI. Lower molecular weight bands indicate CGCG sequences that were methylated. Bottom panels show bisulfite sequencing results in breast cancer cells treated with DMSO or rotenone. *D*, median beta values from 533 TCGA breast tumors analyzed on the Infinium HumanMethylation450 BeadChip platform show the methylation status at nine CpG loci across the miR-663 promoter region. Each locus on the bead chip array is denoted by its distance from the beginning of the miR-663 precursor transcript. \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ . Real-time PCR data represent the average of three biological replicates. Error bars represent S.D. Error bars for tumor methylation beta values are S.E.

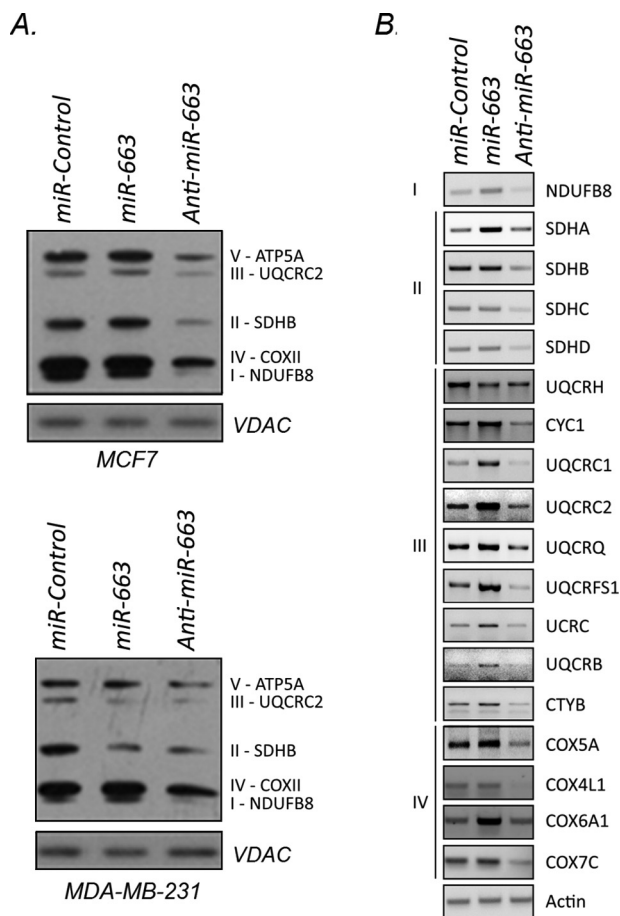
ulation of miR-663 resulted in broad transcriptional reprogramming of Complexes I–IV respiratory subunits (Fig. 4*B*). The anti-miR-663 reduced the expression of most subunits assayed; in particular NDUFB8 (Complex I), SDHB and SDHC (Complex II), UQCRC1 and UQCRC2 (Complex III), and COX4L1 and COX7C (Complex IV). Ectopic expression of miR-663 resulted in increased expression of several of those genes. We conclude that miR-663 regulates nuclear-encoded respiratory chain subunits involved in Complexes I–IV.

#### miR-663 regulates expression of OXPHOS assembly factors and stability of supercomplexes

In addition to relying on the expression of OXPHOS subunits, the mitochondrial function requires assembly factors for the construction of OXPHOS complexes (29, 30). We tested the

role of miR-663 in regulating key assembly factors. We found that some assembly factors for Complexes I–IV were reprogrammed at the transcriptional level after modulation of miR-663 (Fig. 5*A*). The anti-miR-663 reduced the expression of nearly all assembly factors assayed, including NDUFAF1 (Complex I), SDHAF2 (Complex II), UQCC2 (Complex III), and SCO1 (Complex IV). Ectopic expression of miR-663 increased expression of several of those genes and others (Fig. 5*A*). Next, we used luciferase assay to test if miR-663 directly targeted any of the dysregulated respiratory genes. Using the miRNA target prediction software miRanda (<http://www.microrna.org/microrna/home.do>)<sup>3</sup>, we identified SDHAF2 and UQCC2 as

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**Figure 4. Inhibition of miR-663 reprograms OXPPOS gene expression.** A, protein expression of subunits for each OXPPOS complex in mitochondria from miR-663-modulated MCF7 cells (top blot) and MDA-MB-231 cells (bottom blot). B, semi-quantitative PCRs of respiratory complex subunits using mRNA isolated from miR-663-modulated MCF7 cells. All PCRs were run at the same time as their corresponding actin controls and represent two biological replicates.

potential targets of miR-663. We tested these two predicted targets and one unpredicted Complex I subunit, NDUFB8 (regulated by miR-663). We found that miR-663 directly interacts with the 3'-UTR of UQCC2 (Fig. 5B). These data demonstrate that miR-663 regulates the respiratory chain directly by modulating the expression of a Complex III assembly factor; importantly we found no evidence that miR-663 localizes to mitochondria (supplemental Fig. S3).

Large macromolecular OXPPOS complexes, or supercomplexes, are assembled from smaller complexes and are important for electron transfer between complexes (31, 32). These supercomplexes are thus an important factor in mitochondrial function (33). We used Blue Native (BN)-PAGE to assess the role of miR-663 in supercomplex stability. In accord with the subunit and assembly factor expression changes, we observed a robust destabilization of OXPPOS supercomplex Complex I/ComplexIII<sub>2</sub>/ComplexIV, ComplexIII<sub>2</sub>/IV, ComplexIII<sub>2</sub>, and ComplexII in MCF7 cells by the anti-miR-663 (Fig. 5C). In MDA-MB-231 cells, we observed destabilization of ComplexIII<sub>2</sub> and ComplexII (Fig. 5C). Ectopic expression of miR-663 increased the expression of many subunits and assembly factors without affecting the stability of supercomplexes.

Unexpectedly, ectopic expression of miR-663 reduced the stability of the ComplexIII<sub>2</sub> in both MCF7 and MDA-MB-231 lines.

To determine the impact of subunit and assembly factor reprogramming on supercomplex assembly, we used CRISPRs to disrupt many miR-663-target subunits and assembly factors and assessed the effects by BN-PAGE. Genetic disruptions were confirmed by heteroduplex mobility assay (HMA) or Western blotting (supplemental Fig. S4). Disruption of each subunit or assembly factor resulted in complex-specific disruption of supercomplexes (Fig. 5D). Disruption of the Complex I subunit NDUFB8 destabilized ComplexI/ComplexIII<sub>2</sub>/ComplexIV and ComplexI/ComplexIII<sub>2</sub>. Disruption of the Complex II assembly factor SDHAF2 destabilized Complex II. Disruption of the Complex III assembly factor UQCC1 destabilized ComplexIII<sub>2</sub>/IV and ComplexIII<sub>2</sub>. Disruption of the Complex IV assembly factor COX10 destabilized ComplexIII<sub>2</sub>/ComplexIV and increased ComplexIII<sub>2</sub> and ComplexIV. Furthermore, except for SDHAF2, disruption of each of these OXPPOS genes reduced miR-663 expression by a feedback mechanism (Fig. 5E).

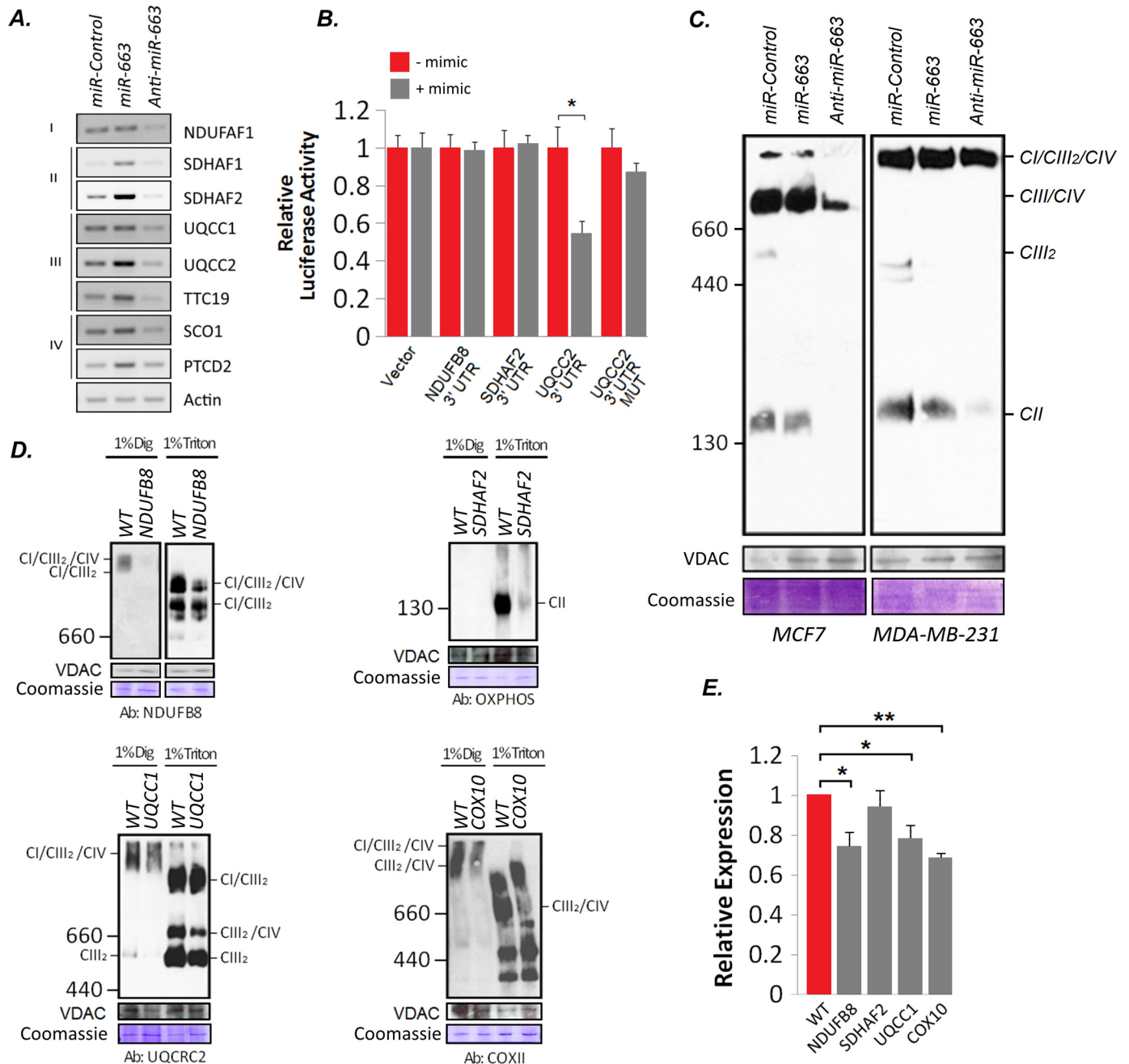
**miR-663 regulation of OXPPOS gene expression modulates complex activity**

The expression of OXPPOS subunits, their assembly factor-mediated construction into larger complexes, and the stability of those complexes determine, in part, the enzymatic activity of each complex (34). Except for Complex IV, the anti-miR-663 reduced the activity of all OXPPOS complexes (Fig. 6). In most cases, ectopic expression of miR-663 did not alter the enzyme activity, except Complex II. Complex III activity was reduced by ectopic expression of miR-663, which may be a result of the observed reduction in UQCRH expression and the destabilization of ComplexIII<sub>2</sub> after expressing miR-663. These results indicate that miR-663 regulates OXPPOS complex activity by mediating OXPPOS gene expression and complex assembly.

**miR-663 regulates tumorigenic properties in vitro and tumor growth in vivo**

Mitochondrial dysfunction is a hallmark of cancer (7, 9, 35). Because miR-663 regulates mitochondrial function, we tested the impact of miR-663 on *in vitro* tumorigenic properties and *in vivo* tumorigenesis. Ectopic expression of miR-663 decreased cellular invasion (Fig. 7A) in both MCF7 and MDA-MB-231 cells *in vitro* but did not affect *in vitro* proliferation (Fig. 7B). Consistent with *in vitro* studies, we demonstrated increased tumor growth rate in anti-miR-663-expressing xenografts and decreased growth rate in miR-663-expressing xenografts compared with the control (Fig. 7C). Tumor weight demonstrated the same relationship; the anti-miR-663 and ectopic expression increased and decreased tumor weight, respectively (Fig. 7D). Based on these studies, we conclude that miR-663 suppresses breast tumorigenesis.

We expanded our analysis into the TCGA database and found that miR-663 expression correlates with stage-specific survival in breast tumors. miR-663 was elevated in stage II tumors. Stage II tumors are characterized by being smaller



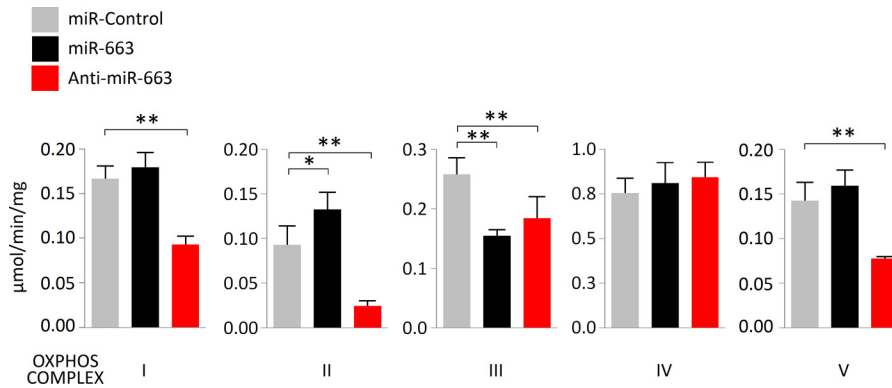
**Figure 5. Inhibition of miR-663 destabilizes supercomplexes.** *A*, semi-quantitative PCRs of respiratory complex assembly factors using mRNA isolated from miR-663–modulated MCF7 cells. All PCRs were done at the same time as their corresponding actin control. *B*, luciferase assays assessing direct interactions between miR-663 and the 3′-UTRs of predicted miR-663 targets. *C*, BN-PAGE of OXPHOS supercomplexes in MCF7 cells (*left blot*) and MDA-MB-231 cells (*right blot*) stably expressing either a microRNA control vector, an miR-663 expression vector, or an anti-miR-663 vector. Mitochondria were solubilized in 1% digitonin. Blots were probed with an OXPHOS antibody mixture (MitoSciences) including antibodies against NDUFB8 (Complex I), SDHB (Complex II), UQCRC2 (Complex III), COXII (Complex IV), and ATP5A (Complex V). *D*, Blue Native blots showing supercomplex stability after CRISPR disruption of subunits or assembly factors for Complexes I–IV in MCF7 cells. Mitochondria were solubilized in 1% digitonin or 1% Triton as noted. Antibodies are indicated below each blot and dilutions of 1:1000 were used for all. *E*, primary miR-663 transcript expression by real-time PCR using cDNA prepared from CRISPR transfected MCF7 cells. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ . BN-PAGE and semi-quantitative PCR data are representative of two biological replicates. Real-time PCR data represent three biological replicates. Luciferase assays represent two biological replicates run in quadruplicate. Error bars depict S.D.

than 5 cm with lymph node involvement, or larger than 5 cm with no node involvement. Interestingly, miR-663 expression imparted a positive survival effect (Fig. 7E). We also found miR-663 to be elevated at metastatic stage M0 (no detectable metastases) where it also imparted a positive survival effect (Fig. 7F). These data suggest that miR-663 plays an important role in early stage breast tumor biology.

## Discussion

Mitochondria play a regulatory role in tumorigenesis, evidenced by their connection with tumor suppressor function (36) and apoptosis (37). Furthermore, mitochondrial dysfunction induces the Warburg phenomenon and metabolic reprogramming (37, 38). Although it is well-appreciated that

## Retrograde miR-663 suppresses tumorigenesis



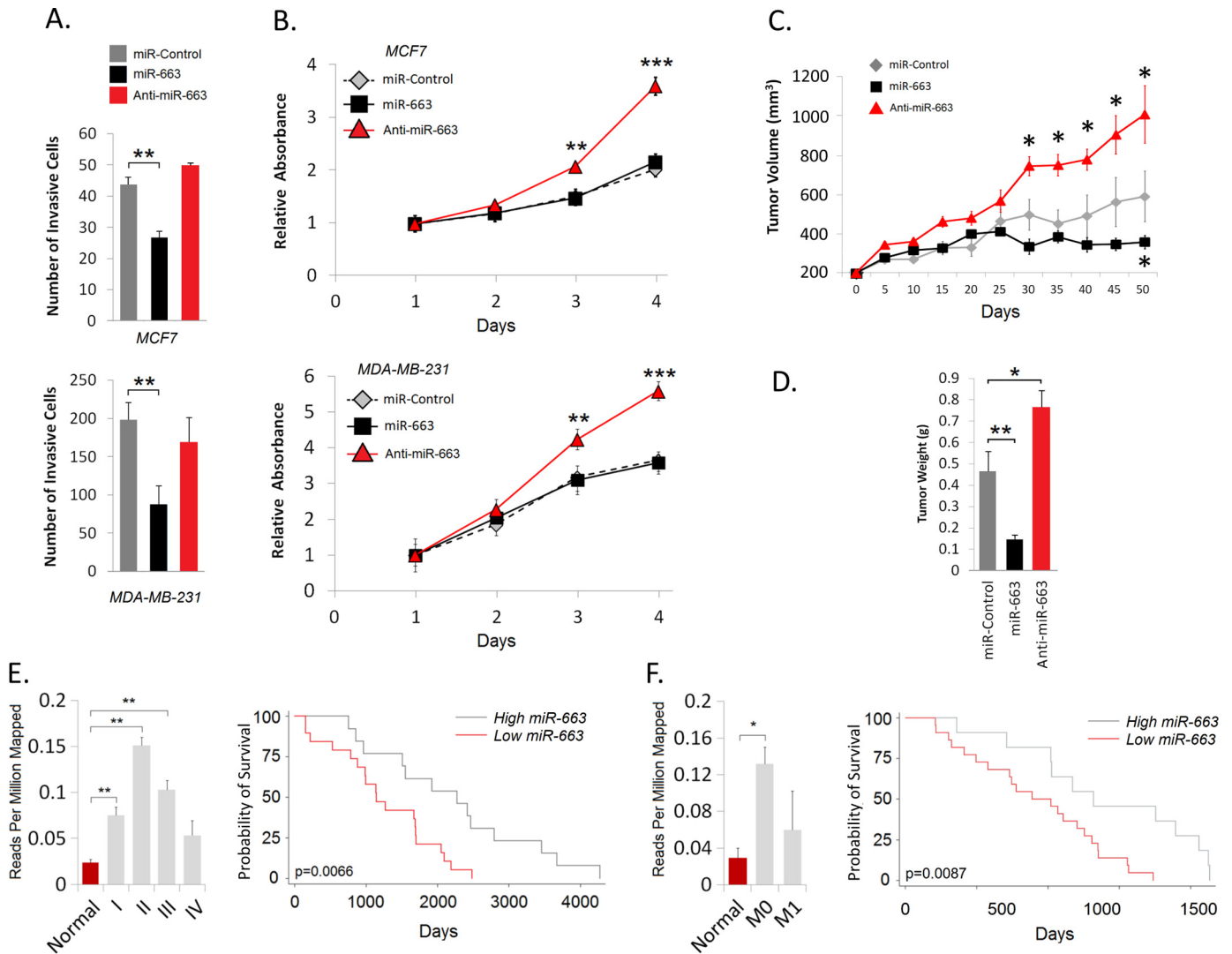
**Figure 6. Inhibition of miR-663 alters OXPHOS enzyme activity.** Isolated mitochondria were used to conduct enzymatic activity assays of OXPHOS Complexes I–V in MCF7 cells expressing a microRNA control vector, an miR-663 expression vector, or an anti-miR-663 vector. Activities were normalized to total protein. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ . Assays represent two biological replicates run in triplicate. Error bars depict S.D.

mitochondrial dysfunction plays an important role in tumorigenesis, the mechanisms underlying this are not fully understood. Retrograde signaling is a response to mitochondrial dysfunction in which nuclear gene transcription is reprogrammed through signaling cascades mediated by second messengers such as ROS and cytosolic calcium. Our knowledge of the mechanisms by which these alterations occur is currently incomplete; our understanding of the role of miRNA in retrograde signaling is particularly lacking. Among other processes, miRNAs have been shown to regulate mitochondrial function. miR-30 targets the p53-Drp1 axis, whereas miR-181c translocates into mitochondria to directly modulate gene expression (39, 40). Current research describes an increasingly complex interplay of nuclear-encoded miRNAs that localize to mitochondria, perhaps joining a pool of miRNAs that are transcribed by mtDNA itself (41–43). miRNAs, therefore, appear poised to modulate mitochondria-to-nucleus communication, which could play an important role in cancer. In this study, we identified miR-663 as an important regulator of the mitochondrial retrograde response and tumorigenesis.

The miR-663 gene family is a primate-specific group of miRNAs of which only miR-663a (or miR-663) and miR-663b have been identified in humans. The role of miR-663 in tumorigenesis appears to be tissue specific; in papillary thyroid carcinoma (25), glioblastoma (44, 45), and pancreatic cancer (46), miR-663 suppresses tumorigenic characteristics, whereas in nasopharyngeal carcinoma (47), lung cancer (48), and prostate cancer (49), miR-663 induces tumorigenic characteristics. Furthermore, miR-663 expression is decreased in pediatric acute myeloid leukemia patients (50), whereas its overexpression was associated with chemoresistance in breast tumors (51). The complexity of miR-663's role in tumorigenesis is at least partly because of the complex nature of its target genes. On the one hand, miR-663 targets oncogenes like H-Ras (52), PIK3CD (44), and CXCR4 (45). H-Ras is highly mutated in bladder cancer (53) and is a driving factor in breast cancer (54, 55). PIK3CD is ubiquitously expressed in follicular lymphoma and is overexpressed in many solid tumors (56, 57); it regulates cell chemotaxis in breast cancer (58). On the other hand, miR-663 targets the tumor suppressor APC (59, 60) which is known to be frequently modulated epigenetically in breast cancer and regulates the Wnt/ $\beta$ -catenin pathway (61, 62). Furthermore, several

miR-663 targets are known to switch between their tumor suppressive and oncogenic functions depending on the stage of cancer (e.g. TGF- $\beta$ 1) (24, 26, 48, 63) or their genetic context (e.g. p21 (47) and JunD (27)). It is therefore important to characterize the role of miR-663 in other cellular and tumorigenic contexts. From an miRNA microarray, we identified miR-663 and a number of other miRNAs that were dysregulated in Rho<sup>0</sup> cells and restored to parental expression levels after restoring mtDNA, presenting us with reversible, candidate mediators of retrograde communication. We chose miR-663 for further investigation for several reasons. Firstly, its role in breast cancer remains unclear. Secondly, the miR-663 promoter has been shown to be labile to DNA methylation. This, in conjunction with our previous results showing that mitochondrial dysfunction alters nuclear DNA methylation, highlights miR-663 as a potentially important factor in understanding this mechanism in breast cancer. We validated the dysregulation of miR-663 in Rho<sup>0</sup> cells by real-time PCR (Fig. 1B) and showed that it is dysregulated by mitochondrial dysfunction (Fig. 1C). We demonstrated that miR-663 is regulated by ROS (Fig. 1, D and F). This is supported by previous reports showing that oxidative stress alters miR-663 expression (27, 64, 65).

Our previous studies demonstrated a link between mitochondrial function and nuclear DNA methylation (19, 66, 67). Here we expanded our studies to elucidate this mechanism and identify its targets. The miRNAs that were dysregulated in our screen returned to parental levels upon restoration of mtDNA (Fig. 1A). We observed that mitochondrial dysfunction resulted in the reduction of both the primary and mature miR-663 molecules (Fig. 1C). Our previous study showing the reversal of epigenetic changes by replenishing mtDNA indicated that these miRNA forms were epigenetically regulated. We demonstrated that mitochondrial dysfunction (pharmacological inhibition of OXPHOS complexes or removal of mtDNA) hypermethylated the miR-663 promoter and down-regulated its expression (Fig. 2, A and B). Hypermethylation and down-regulation of miR-663 was not observed in the presence of 5-aza-2'-deoxycytidine (Fig. 2C), confirming the methylation mechanism. Total DNA methyltransferase activity was elevated in cells treated with complex inhibitors (Fig. 2D), indicating that mitochondrial dysfunction alters methylation of miR-663 and other miRNAs identified in this study by modulating the activ-



**Figure 7. miR-663 regulates cellular growth, invasion, and tumor progression.** A and B, after stable transfection of MCF7 and MDA-MB-231 breast cancer cell lines with the microRNA control, miR-663 expression vector, or an anti-miR-663 vector, tumorigenic assays were carried out to assess the role of miR-663 in cellular invasion (A) and cellular proliferation (B). C and D, tumor growth (C) and tumor weight (D) were assessed in mouse subcutaneous xenografts stably expressing a microRNA control, miR-663 expression vector, or an anti-miR-663 vector. E, analysis of TCGA tumors (bar graph) showing mature miR-663 expression by tumor stage I–IV (tumor sample  $n = 82, 121, 390, 150,$  and  $13$  for normal, stage I, stage II, stage III, and stage IV, respectively). Kaplan-Meier curves (right plot) derived from TCGA data showing 10-year survival of patients with group stage II breast tumors, stratified by high ( $n = 15$ ) or low ( $n = 18$ ) mature miR-663 expression. F, analysis of TCGA tumors (bar graph) showing mature miR-663 expression by tumor metastatic stage (tumor sample  $n = 82, 690,$  and  $15$  for normal, M0, and M1, respectively). Survival of patients (right plot) with metastatic stage 0 breast tumors stratified by high ( $n = 11$ ) and low ( $n = 22$ ) mature miR-663 expression. Patients were stratified by high (expression values  $>0$ ) and low (expression values of 0) mature miR-663 expression. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Cellular invasion and proliferation assays present at least two biological replicates. Proliferation assays were run in quadruplicate. *In vitro* assay error bars represent S.D. Xenograft and primary tumor error bars denote S.E.

ity of DNA methyltransferases. These results are consistent with a previous report showing that depletion of mtDNA increases expression of DNMT1 and methylation of the promoters of MGMT, EDNRB, and CHD1 (68).

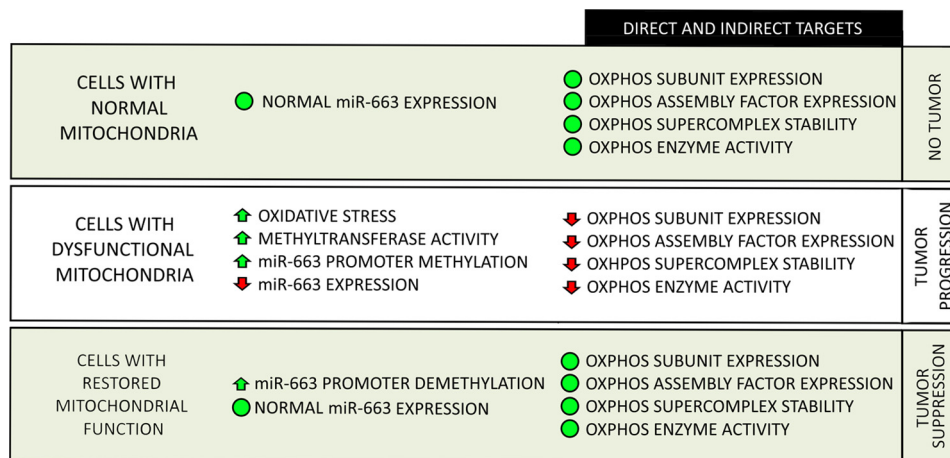
Mitochondrial dysfunction also hypermethylated the promoter and reduced the expression of miR-663 in breast cancer cell lines (Fig. 3, C and D). Using the TCGA database, we observed hypermethylation of the miR-663 promoter in breast tumors (Fig. 3D), as also reported previously (69). Expression analysis of DNA methylation modifying enzymes in the TCGA database showed that the tumors with hypermethylated miRNA-663 also demonstrate elevated expression of the DNA methylases DNMT1, DNMT3A, DNMT3B, and DNMT3L and reduced expression of the DNA demethylases ALKBH3 and

FTO (supplemental Fig. S1). This supports our *in vitro* work suggesting a shift in the methylation machinery favoring hypermethylation. Considering that low expression of miR-663 correlated with reduced patient survival in the same tumors (Fig. 7, E and F), DNA methylation of miR-663 appears to be a significant event in breast cancer. Our previous study demonstrated that DNA methylation is a broad mechanism of mitochondrial regulation of nuclear transcription (19), and our current study defines miR-663 as the first miRNA regulated by this mechanism.

In this study, we report that miR-663 positively regulates expression of OXPHOS subunit genes and genes involved in the assembly of OXPHOS complexes. We demonstrated the direct regulation of UQCC2 by miR-663 using luciferase assays.



## Retrograde miR-663 suppresses tumorigenesis



**Figure 8. miR-663 retrograde regulation of OXPHOS and its role in tumorigenesis.** A summary figure explains the regulatory mechanism underlying retrograde regulation of miR-663 and its role in controlling mitochondrial function and tumorigenesis.

Although an indirect correlation between an miRNA and its target genes is most common, several alternative regulatory mechanisms have been recently observed in which an miRNA can stabilize its target genes or activate their promoters thereby increasing target transcript abundance (70). Interestingly, we found that miR-663 ectopic expression stabilized the UQCC2 transcript and the anti-miR-663 destabilized it (Fig. 5A). Mutations in UQCC2 destabilize both Complex I/III<sub>2</sub> and Complex III<sub>2</sub>/IV (71). They also decrease Complexes I, III, and IV enzymatic activity. The anti-miR-663 in our study induced all of these defects in MCF7 cells, suggesting that UQCC2 alone may explain many of the mitochondrial defects that we observed after inhibiting miR-663.

We show that up-regulation of mitochondria-dependent miR-663 in breast cancer cell lines decreased Matrigel invasion; antisense inhibition of miR-663 increased cellular proliferation (Fig. 7, A and B). We demonstrate the same role for miR-663 *in vivo*. We show that xenografts of MCF7 cells stably transfected with the anti-miR-663 form larger tumors than xenografts of control transfected cells (Fig. 7, C and D). Conversely, miR-663 ectopic expression reduced tumor volume. In agreement, TCGA survival analyses show that breast cancer patients with stage II or M0 tumors with high miR-663 expression had increased survival compared with those with low miR-663-expressing tumors (Fig. 7, E and F). Collectively our data indicate that miR-663 down-regulation promotes breast tumorigenesis, whereas up-regulation inhibits it.

In conclusion, we established the role of miR-663 in controlling mitochondria-to-nuclear cross talk that plays a crucial role in regulating mitochondrial function (see Fig. 8 for a summary). Briefly, cells containing normal mitochondria and normal miR-663 expression show proper expression of OXPHOS subunits, assembly factors, stable OXPHOS supercomplexes, and normal OXPHOS enzyme activities. However, dysfunctional mitochondria induce oxidative stress, increase methyltransferase activity leading to a hypermethylated miR-663 promoter and reduced miR-663 expression. Inhibition of miR-663 expression reduces OXPHOS gene expression, destabilizes the supercomplexes, and reduces OXPHOS enzymatic activities which together contribute to increased tumorigenesis. Restoration

of mitochondrial function reverses these changes, thereby inhibiting tumor progression. Taken together our studies highlight miR-663 as a central player in controlling mitochondria-to-nucleus retrograde communication and development of cancer.

### Experimental procedures

#### Cell culture

Cell lines were purchased from American Type Culture Collection (ATCC) (Manassas, VA), except for the 143B Rho<sup>0</sup> line which was a gift from Dr. James R. Smiley. The Rho<sup>0</sup> cells were generated genetically by transfecting parental cells with a mitochondrially-targeted nuclease, UL12.5 (72). The cybrid line was derived from the genetically-created Rho<sup>0</sup> line as described previously (73). MCF7 and MDA-MB-231 cells were grown in DMEM supplemented with 10% fetal bovine serum (Mediatech, Inc., Manassas, VA) and 0.1% penicillin/streptomycin (Mediatech). The generation and culture conditions of the MCF7 cells with tetracycline-inducible dominant negative POLG D1135A were described previously (74). POLG D1135A was induced by culturing the cells in 1000 ng/ml doxycycline for 10 days. All 143B-derived lines were maintained in DMEM (Mediatech) with 10% fetal bovine serum (Mediatech) and 0.1% penicillin/streptomycin supplemented with 50 μg/ml uridine (Sigma) and 100 μg/ml pyruvate (Sigma). Cells were maintained at 37 °C, 95% humidity, and 5% carbon dioxide.

#### Chemical treatment

5-aza-2'-deoxycytidine, rotenone, antimycin A, and oligomycin were delivered in DMSO. Malonate and potassium cyanide were delivered in water. All chemicals were purchased from Sigma.

#### Antibodies

Mouse monoclonal antibodies, NDUFB8 (ab110242), UQCRC2 (ab14745), COXII (ab110258), and OXPHOS mixture (ab110413) were obtained from MitoSciences and used at dilutions of 1:1000 for all.

### miRNA microarray

One RNA sample from parental, Rho<sup>0</sup>, and cybrid (repleted mtDNA) cells was analyzed by deCODE Genetics (Reykjavik, Iceland) for expression of 661 miRNAs using the miRCURY LNA<sup>TM</sup> array (v.9.2) (Exiqon A/S, Vedbaek, Denmark). After RNA passed quality control by the Bioanalyzer 2100 and Nano-Drop, it was labeled using the miRCURY Hy3<sup>TM</sup>/Hy5<sup>TM</sup> power labeling kit and hybridized on the array. The quantified signals for the background correction were normalized using the global LOWESS (locally weighted scatterplot smoothing) regression algorithm. Three array slides were used; each one hybridized the Rho<sup>0</sup> cDNA to the green channel (Hy3) and hybridized cDNA from either the parental or the cybrid line to the red channel (Hy5). Expression of miRNAs was compared between samples by comparing Hy3 and Hy5 signals.

### Semi-quantitative RT-PCR and real-time PCR

RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer's specifications. RNA was DNase treated (Promega, Madison, WI) and reverse transcribed using iScript<sup>TM</sup> cDNA synthesis kit from Bio-Rad according to manufacturer's specifications. Semi-quantitative RT-PCR was conducted using Promega's GoTaq<sup>®</sup> Green Master Mix. Unless otherwise stated, reactions were carried out for 32 cycles. Real-time PCR was conducted with Bio-Rad IQ<sup>TM</sup> SYBR Green Supermix and analyzed using the Roche LightCycler 480. Data were derived from three independent RNA samples. Semi-quantitative data are representative of two replicates.

### Methylation-specific PCR, bisulfite genomic sequencing, and COBRA analysis

Genomic DNA was isolated using TRI Reagent and bisulfite converted using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). Methylation-specific PCR and bisulfite sequencing of miR-663 were conducted as described previously (50). Sequencing was done on the Applied Biosystems 3730 DNA Analyzer and analyzed with BiQ Analyzer (<http://biq-analyzer.bioinf.mpi-inf.mpg.de/>)<sup>3</sup>. For COBRA analyses, 10 ng of bisulfite-converted DNA was amplified using the bisulfite sequencing primers to capture the BstUI cut site. The PCR product was then digested with BstUI for 1 h at 60 °C and run on a 1% agarose gel.

### TCGA data collection

Publicly available data for primary breast tumors were obtained from the TCGA publications ([https://tcga-data.nci.nih.gov/docs/publications/brca\\_2012/](https://tcga-data.nci.nih.gov/docs/publications/brca_2012/))<sup>3</sup>. mRNA expression data were obtained for 522 breast tumors and normal samples. miRNA data normalized as reads per million mapped (RPM) were obtained for 780 breast tumors and normal samples. Methylation data for nine miR-663-associated CpGs were obtained for 552 breast tumors and normal samples.

### Statistical analysis

Statistical analyses were conducted using Stata version 11 (Statacorp LP, College Station, TX). Analysis of variance

(ANOVA) was used to compare multiple means, and multiple comparisons were corrected by the Bonferroni method. Kaplan-Meier curves were compared using the log-rank test. For all tests  $p < 0.05$  was considered significant.

### Lentivirus production and transduction

Lentivirus constructs for miRNA control and miR-663 expression were purchased from Applied Biological Materials (Richmond, BC, Canada), and the anti-miR-663 construct was purchased from System Biosciences (Palo Alto, CA). Transfection of constructs was done using FuGENE HD (Promega) according to manufacturer's specifications. Constructs were co-transfected with packaging plasmids PMD2G and PSPAX2 into 293T cells. Media were filtered and virus precipitated in 10% PEG 8000, dissolved in PBS, and added to target cells in complete media with 5 µg/ml Polybrene. Puromycin (5 µg/ml) was added to select transduced cells.

### Design of CRISPRs

CRISPR primers were designed using E-CRISP (<http://www.e-crisp.org/E-CRISP/designcrispr.html>)<sup>3</sup>. One µl of each 100-µM primer was added to 48 µl of oligo annealing buffer (Promega), boiled 10 min, and cooled to room temperature. Three microliters of annealed primers and 1 µl of *Bbs*I digested pSpCas9(BB)-2A-GFP (5 ng/µl) plasmid were ligated using the LigaFast<sup>TM</sup> rapid ligation system (Promega). CRISPRs were transfected into MCF7 cells. Positive cells were FACS sorted on GFP and plated to grow in single colonies. Colonies were selected and grown for screening by Western blotting.

### Luciferase assays

Primers were designed to incorporate the entire 3'-UTR of the gene of interest, and restriction sites for NheI (5') and SbfI (3') were added. 3'-UTRs were amplified from MCF7 DNA, digested, and cloned into the pmirGLO Dual-Luciferase Vector (Promega). Clones were screened by restriction digest and confirmed by sequencing. Vectors were transfected into MCF7 cells in 96-well plates (100 ng/well) in the absence or presence of a miR-663 mimic (100 nM). Luminescence was analyzed in triplicate on a BioTek Synergy H1 Multi-Mode Reader using the Dual-Glo Luciferase Assay System (Promega). Firefly activity was normalized to *Renilla* activity.

### Blue-Native polyacrylamide gel electrophoresis

Mitochondrial isolation was carried out as described previously (75). BN-PAGE was performed on isolated mitochondria to analyze endogenous protein complexes as described previously with minor modifications (30, 76).

### Heteroduplex mobility assay

Primers were designed to amplify a 150–300-bp region incorporating the CRISPR guide RNA binding site of the target gene. After amplification, HMA assay was carried out as described previously (77).

### MTT assay

In a 96-well plate, 5000 MCF7 or 2000 MDA-MB-231 breast cancer cells were plated per well in 100 µl of growth media.

## Retrograde miR-663 suppresses tumorigenesis

Each cell line was plated in quadruplicate, and iterations of each line were plated for daily analysis. For 4 days, cell viability was quantified as described previously (78).

### Reactive oxygen species quantification

After treatment, cells were resuspended in 10  $\mu\text{M}$  CM-H<sub>2</sub>DCFDA and incubated in the dark at 37 °C for 30 min and washed twice with PBS. Fluorescence of DCF-DA was measured by FACS using a BD LSR II Analyzer. Three replicates of each cell line were analyzed.

### Mouse xenografts

Xenograft experiments were carried out as described previously (78). Female athymic mice (three mice per group; two tumors per mouse) were implanted with 17 $\beta$ -estradiol pellets 2 days before tumor cell injection. A suspension of 1  $\times$  10<sup>8</sup> tumor cells per milliliter was made in a 1:1 mixture of Matrigel and PBS, and 0.1 ml (1  $\times$  10<sup>7</sup> cells) was injected subcutaneously. Tumor growth was monitored externally using calipers for 50 days.

### OXPPOS enzymatic assays

Mitochondria were isolated as described above and resuspended at 1  $\mu\text{g}/\text{ml}$  protein. Enzymatic activities of OXPPOS complexes were measured as described previously (79).

### Matrigel

A cellular invasion was measured using Corning BioCoat Matrigel Invasion Chambers (Bedford, MA) according to the manufacturer's instructions. Invading cells were stained using the Kwik-Diff Kit (Thermo Scientific) according to the manufacturer's protocol. Experiments were done in triplicate.

### DNA methyltransferase activity assay

Whole-cell DNA methyltransferase activity was assessed by DNA Methyltransferase Activity Kit (EpiGentek, Farmingdale, NY). The manufacturer's protocol was used to analyze 20  $\mu\text{g}$  of whole cell lysate. Analyses were done in triplicate.

*Author contributions*—T. C. collected and analyzed data and drafted the manuscript. B. S. assisted in conducting xenografts studies. V. M. assisted in conducting Blue Native gels. P. B. assisted in analyzing OXPPOS activity. K. K. S. conceived, planned, and directed the research project and assisted in writing and editing the manuscript.

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