Mitochondrial complex I activity and NAD+/NADH balance regulate breast cancer progression

Antonio F. Santidrian,1,2 Akemi Matsuno-Yagi,1 Melissa Ritland,1,2 Byoung B. Seo,1 Sarah E. LeBoeuf,1,2 Laurie J. Gay,1,2 Takao Yagi,1 and Brunhilde Felding-Habermann1,2

1Department of Molecular and Experimental Medicine and 2Department of Chemical Physiology, The Scripps Research Institute, La Jolla, California, USA.

Despite advances in clinical therapy, metastasis remains the leading cause of death in breast cancer patients. Mutations in mitochondrial DNA, including those affecting complex I and oxidative phosphorylation, are found in breast tumors and could facilitate metastasis. This study identifies mitochondrial complex I as critical for defining an aggressive phenotype in breast cancer cells. Specific enhancement of mitochondrial complex I activity inhibited tumor growth and metastasis through regulation of the tumor cell NAD+/NADH redox balance, mTORC1 activity, and autophagy. Conversely, nonlethal reduction of NAD+ levels by interfering with nicotinamide phosphoribosyltransferase expression rendered tumor cells more aggressive and increased metastasis. The results translate into a new therapeutic strategy: enhancement of the NAD+/NADH balance through treatment with NAD+ precursors inhibited metastasis in xenograft models, increased animal survival, and strongly interfered with oncogene-driven breast cancer progression in the MMTV-PyMT mouse model. Thus, aberration in mitochondrial complex I NADH dehydrogenase activity can profoundly enhance the aggressiveness of human breast cancer cells, while therapeutic normalization of the NAD+/NADH balance can inhibit metastasis and prevent disease progression.

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Results

Enhancement of complex I activity in human breast cancer cells inhibits tumor growth and metastasis. Complex I function in MDA-MB-435 and MDA-MB-231 cells was enhanced by stable transduction with Ndi1. The encoded enzyme was expressed by 90% of the cells, localized to mitochondria (Figure 1A) without altering the stoichiometry of mitochondrial complexes (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI64264DS1), and significantly enhanced mitochondrial respiration in the intact tumor cells (Figure 1B). Confirming the functional contribution of Ndi1, respiration in Ndi1-expressing MDA-MB-435 and MDA-MB-231 cells was inhibited by the mammalian complex I antagonist rotenone by only 20% and 28%, respectively, in contrast to 85% and 84% in controls. In all cases, oxygen consumption was fully blocked by the complex III inhibitor antimycin A (Figure 1B), which indicates that the measurements specifically reported mitochondrial respiration. Ndi1 also increased OXPHOS-uncoupled respiration, as shown by the oligomycin-inhibited respiratory rate (Supplemental Figure 2A), and enhanced complex I-mediated respiration in permeabilized cells upon addition...
of the complex I substrates malate and glutamate (Supplemental Figure 2, B and C). Ndi1 did not change the maximal capacity of the mitochondrial electron transfer system (Supplemental Figure 2A). Resistance to rotenone and susceptibility to flavone affirmed the contribution of Ndi1 (Supplemental Figure 2, B and C). In both tumor cell models, Ndi1 did not alter mtDNA content or mitochondrial membrane potential (Figure 1C). Reflecting overall energy metabolism and balance between glycolysis and mitochondrial OXPHOS, Ndi1 expression decreased ATP levels, whereas lactate production was unchanged in both breast cancer cell models (Figure 1C). These results demonstrated full functional integration of Ndi1 NADH dehydrogenase into the respiratory chain of 2 human cancer cell models, causing enhanced mitochondrial complex I activity without a major effect on overall energy balance.

To investigate whether enhancement of tumor cell complex I activity affects tumorigenicity and metastasis, we first analyzed effects on mouse mammary fat pad tumors. Ndi1 expression inhibited mammary fat pad tumor growth of MDA-MB-435 and MDA-MB-231 cells, whereas control cells transduced with empty vector exhibited a normal growth rate (Figure 2, A and B). Ndi1 expression inhibited lung colonization (experimental metastasis) by MDA-MB-435 or MDA-MB-231 cells after i.v. injection (Supplemental Figure 2). Ndi1 expression inhibited multorgan experimental metastasis, as indicated by noninvasive bioluminescence imaging 7 weeks after i.v. injection of 2.5 \times 10^6 MDA-MB-435 control or Ndi1-expressing cells (n = 5). (D) Knockdown of complex I subunit NFUFV1 expression inhibited complex I activity and respiratory capacity in MDA-MB-435 cells. NDUFV1-knockdown (shV1) and control (shCT) cells were compared. Complex I was immunocaptured from cell lysates, analyzed based on oxidation of NADH to NAD+, and expressed as mean OD/min/mg protein (n = 3). Routine mitochondrial respiration, corrected for residual oxygen consumption due to oxidative side reactions, was measured in intact MDA-MB-435 control and NDUFV1-knockdown cells by high-resolution respirometry (n = 3). (E) NDUFV1 knockdown increased lung colonization activity in MDA-MB-435 cells. NDUFV1-knockdown and control cells were compared (n = 8). Data are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, nonparametric Mann-Whitney test (A, B, and E) or unpaired 2-tailed Student's t test (D).

To corroborate a role of aberrant complex I activity in metastatic progression, we challenged our findings with an independent reverse approach, asking whether interference with tumor cell complex I function enhances metastatic aggressiveness. To this end, we knocked down NDUFV1 in MDA-MB-435 cells. NDUFV1 encodes the 51-kDa complex I subunit, which belongs to the minimal assembly required for catalysis and carries the NADH binding site (26). Mutations of this gene cause complex I deficiency in severe neurological diseases such as Leigh syndrome (26). NDUFV1 knockdown (Supplemental Figure 3) reduced complex I activity by 92% and respiratory capacity by 51% and significantly enhanced the metastatic activity of the already aggressive MDA-MB-435 cell line (Figure 2, D and E).

These results provided definitive evidence that specific modulation of tumor cell complex I function can significantly alter tumor growth and metastatic activity. Therefore, complex I mutations found in primary tumors of breast cancer patients (6, 11–14, 16) may play a key role in disease progression.

Inhibition of metastatic activity by enhancement of complex I function depends on autophagy. To investigate how complex I activity affects tumor growth and metastasis, we first analyzed effects...
Figure 3
Mitochondrial complex I activity regulates mTORC1 and autophagy. (A) Ndi1 expression enhanced resistance to glucose deprivation in MDA-MB-231 cells, shown after 72 hours of incubation in medium with 5 versus 1 mM glucose. Viability was measured by flow cytometry (non–sub-G0/G1 population). n = 3 independent analyses. *P < 0.05, unpaired 2-tailed Student’s t test. (B) Ndi1 expression influenced mTORC1 activity and autophagy. Western blot analysis for p62, phospho-AKT substrates, and the mTORC1 kinase–related substrates phospho-S6Ser240/244 and phospho-4EBPThr37/46 in MDA-MB-435 or MDA-MB-231 control and Ndi1-expressing cells. β-Tubulin was used as protein loading control. Signal quantification, measured by infrared imaging (total of detectable bands) and expressed relative to control, is shown below. Results are representative of 5 independent experiments. Lanes were run on the same gel but were noncontiguous (white lines). (C) H&E staining and p62, Ki67, or Ndi1 expression in mammary fat pad tumors 5 weeks after implanting 2.5 × 10^5 MDA-MB-435 control versus Ndi1-expressing cells into SCID mice. 2 representative tumors of 6 are shown per group. Original magnification, ×10. (D) Inhibition of complex I activity through NDUFV1 knockdown affected mTORC1 activity and p62 elimination. Western blot analysis for p62, phospho-AKT substrates, phospho-S6Ser240/244, and phospho-4EBPThr37/46, comparing NDUFV1-knockdown versus control MDA-MB-435 cells. Signal quantification, measured by infrared imaging (total of detectable bands) and expressed relative to control, is shown below. Results are representative of 3 independent experiments. Lanes were run on the same gel but were noncontiguous (white lines).
of Ndi1 expression on tumor cell viability and growth. While Ndi1 did not affect proliferation in vitro (Supplemental Figure 4, A and B), Ndi1 increased resistance to glucose deprivation in MDA-MB-231 cells (Figure 3A) and suppressed tumorigenicity in both cell lines (Figure 2A). A similar paradoxical phenomenon was previously described and linked to the ability of tumor cells to undergo autophagy (27–29), without indication at the time that mitochondrial complex I might be involved. Following the hypothesis that breast cancer cell complex I activity can affect autophagy, we analyzed the effects of Ndi1 expression on p62, a ubiquitin-binding scaffolding protein that is eliminated during autophagy (27, 30). Ndi1 expression increased p62 degradation (Figure 3B), which indicated that enhancement of mitochondrial activity induced autophagy. Following signaling pathways that regulate autophagy, we analyzed effects of Ndi1 on mTORC1 activity and AKT, one of mTOR’s main regulators. mTORC1 activity was measured by phosphorylation of mTORC1-related substrates S6 and 4EBP1; AKT activity was based on levels of phospho-AKT substrates. Ndi1 clearly reduced AKT activity in MDA-MB-231 cells and inhibited mTORC1 in both cell lines (Figure 3B), which suggested that complex I activity can induce autophagy by regulating mTORC1 signaling. This process likely involves AKT in MDA-MB-231 cells and alternative mechanisms in MDA-MB-435 cells.

Importantly, levels of p62 and Ki67 were strongly decreased in primary tumors expressing Ndi1 (Figure 3C), which indicates that enhancement of complex I increases tumor cell autophagy and inhibits proliferation in vivo. This conclusion was strongly supported by enhancement of AKT and mTORC1 activities and clear inhibition of p62 elimination by NDUFV1 knockdown, which reduced complex I functionality in the tumor cells (Figure 3D) without affecting their proliferation (Supplemental Figure 4C). These results demonstrated that tumor cell complex I activity can regulate tumor cell mTORC1 signaling and autophagy in vitro and in vivo.

To analyze directly whether complex I–mediated regulation of breast cancer metastasis involves control of tumor cell autophagy, we used ATG5 knockdown (shATG5) to inhibit autophagy in MDA-MB-435 and MDA-MB-231 control and Ndi1-expressing cells, as shown by p62 and LC3BI accumulation. Signal quantification of ATG5, p62 signal, and LC3BI/II ratios, measured by infrared imaging (total of detectable bands) and expressed relative to control, is shown below. β-Tubulin served as protein loading control. Lanes were run on the same gel but were noncontiguous (white lines). (B) ATG5 knockdown blocked the antimetastatic effect of Ndi1 in MDA-MB-435 and MDA-MB-231 cells. Lung colonization was measured by ex vivo lung imaging 7 weeks after i.v. injection of 2.5 × 10^5 tumor cells (n = 8 per group). Boxes denote interquartile range; lines within boxes denote median; whiskers denote minima and maxima. *P < 0.05, nonparametric Mann-Whitney test. (C) ATG5 knockdown enhanced multorgan metastasis and reversed metastasis inhibition by Ndi1 in MDA-MB-435 cells. Shown is noninvasive bioluminescence imaging of 5 representative mice per group at 7 weeks after tail vein injection of 2.5 × 10^5 MDA-MB-435 control or Ndi1-expressing cells, with or without ATG5 knockdown.
agy, we knocked down ATG5, a protein required for autophagy induction (31). Targeting ATG5 in control and Ndi1-expressing MDA-MB-435 and MDA-MB-231 cells by stable transduction with shRNA (Figure 4A and Supplemental Figure 5A) inhibited autophagy, as shown by p62 and LC3BII accumulation (Figure 4A), without affecting proliferation in vitro (Supplemental Figure 5B). Importantly, although basal levels of autophagy facilitated metastasis in MDA-MB-231 cells, ATG5 knockdown abolished the inhibitory effect of Ndi1-mediated complex I enhancement on metastatic organ colonization (Figure 4, B and C). This was seen primarily in the lungs for MDA-MB-231 and in multiple organs for MDA-MB-435, including lung, liver, bone, brain, and adrenal glands. These findings affirm the conclusion that inhibition of metastasis through enhancement of mitochondrial complex I activity depends on autophagy induction in 2 aggressive tumor cell models.

NAD+ level modulation by complex I and NAD+ synthesis and recycling pathways regulate AKT/mTORC1 activity, autophagy, and metastasis. (A) Ndi1 expression enhanced NAD+/NADH balance. NAD+/NADH ratios in whole-cell or mitochondrial extracts of MDA-MB-435 or MDA-MB-231 control versus Ndi1-expressing cells. Ndi1 stabilized NAD+/NADH ratios, especially under metabolic stress induced by glucose deprivation and hypoxia. NAD+/NADH ratios under stress were measured in whole-cell extracts after 48 hours of culture. (B) Interference with NAD+ synthesis and recycling pathways reduced NAD+/NADH ratios. Knockdown of NAMPT (shNAMPT) in MDA-MB-435 and MDA-MB-231 cells decreased NAD+/NADH ratios (whole-cell extracts after 48 hours growth in 5 mM glucose and normoxia). (C) NAMPT knockdown increased lung colonization activity in MDA-MB-435 and MDA-MB-231 cells (n = 6 per group). (D) NAMPT knockdown affected mTORC1 activity and p62 elimination. Western blot analysis for p62, phospho-AKT substrates, phospho-S6Ser240/244, and phospho-4EBPThr37/46 in MDA-MB-435 and MDA-MB-231 NAMPT-knockdown versus control cells. β-Tubulin served as protein loading control. Signal quantification, measured by infrared imaging (total of detectable bands) and expressed relative to control, is shown below. Lanes were run on the same gel but were noncontiguous (white lines). Results are representative of 3 independent experiments. (A–C) Data are mean ± SEM. *P < 0.05, **P < 0.01, unpaired 2-tailed Student’s t test (A and B) or nonparametric Mann-Whitney test (C).

NAD+ level modulation by complex I and alteration in NAD+ synthesis and recycling pathways regulate AKT/mTORC1 activity, autophagy, and metastatic activity. To further investigate the mechanism by which enhancement of complex I activity inhibits tumor progression, we next ruled out alterations in ROS or NADPH production as major underlying causes. Ndi1 expression in MDA-MB-435 and MDA-MB-231 cells did not significantly alter ROS levels or NADPH-reducing equivalents (Supplemental Figure 6, A and B). Likewise, NDUFV1 knockdown did not significantly affect ROS (Supplemental Figure 6C), which suggests that enhancement of complex I activity by Ndi1 inhibits tumorigenicity and metastasis in a ROS-independent manner.

A major function of mammalian complex I and Ndi1 is NADH dehydrogenase activity. Ndi1 expression in MDA-MB-435 and MDA-MB-231 cells increased NAD+/NADH ratios in whole-cell extracts and purified mitochondria, particularly under metabolic
stress induced by glucose or oxygen deprivation (Figure 5A). In culture, an Ndi1-mediated increase in NAD+/NADH ratios was measurable during exponential cell growth (Supplemental Figure 7, A and B). Conversely, disturbance of endogenous complex I activity via NDUFV1 knockdown reduced NAD+/NADH ratios (Supplemental Figure 8). We reasoned that modulation of the cellular redox potential (NAD+/NADH ratio) by mitochondrial complex I activity can regulate the metastatic activity of tumor cells. To test this hypothesis using an independent approach, we experimentally decreased NAD+/NADH ratios in MDA-MB-435 and MDA-MB-231 cells and analyzed the effect on metastasis in vivo.

To decrease the NAD+/NADH ratio in the tumor cells, we disturbed the NAD+ synthesis and recycling pathway by targeting nicotinamide phosphoribosyltransferase (NAMPT). NAMPT is essential for utilization and recycling of nicotinamide (NAM) and for biosynthesis of NAD+ (32). Interference with NAMPT expression through stable transduction with shRNA (Supplemental Figure 9A) reduced cellular NAD+/NADH ratios (Figure 5B), independently of the growth state of the tumor cells (Supplemental Figure 10, A and B) and without affecting cell proliferation in vitro (Supplemental Figure 9B). In vivo, however, NAMPT knockdown significantly enhanced metastasis in MDA-MB-435 and MDA-MB-231 cells (Figure 5C), indicative of a cause-and-effect relationship between reduced NAD+/NADH ratios and metastatic activity. The increased metastatic activity due to interference with cellular NAD+/NADH ratios involved altered mTORC1 activity and autophagy. Western blot analysis for p62, phospho-AKT substrates, and phospho-S6Ser240/244 in MDA-MB-435 or MDA-MB-231 parental cells with or without 48 hours of treatment with 10 mM NIC or NAM. β-Tubulin served as protein loading control. Signal quantification, measured by infrared imaging (total of detectable bands) and expressed relative to control, is shown below. Results are representative of 3 independent experiments. *P < 0.05, **P < 0.01, unpaired 2-tailed Student’s t test (A) or nonparametric Mann-Whitney test (B and C).

Although our results showed that downregulation of NAMPT expression enhanced metastatic activity, chemical NAMPT inhibitors have previously been suggested for anticaner therapy (33). To investigate this potential discrepancy, we analyzed effects of FK866, a noncompetitive inhibitor of NAMPT (34), in vitro and in vivo. In vitro, FK866 decreased cellular NAD+/NADH ratios in MDA-MB-435 and MDA-MB-231 cells to levels similar to those seen after NAMPT knockdown (Supplemental Figure 11A), indicative of an effect of this drug on NAD metabolism. Treatment of NAMPT-knockdown cells with FK866 reduced NAD+/NADH levels further, likely due to a dose effect of NAMPT inhibition (Sup-
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We then asked whether this therapeutic approach might transiently modulate mTORC1 activity regulation, which suggests that NAM treatment strongly interfered with oncogene-driven breast cancer progression (Figure 8C and D, and Supplemental Figure 17). We did not detect effects on mTORC1 activity regulation, which suggests that NAM treatment in the drinking water might transiently modulate mTORC1 activity within PyMT mammary tumors in vivo.

To challenge the concept that NAD⁺ precursor treatment can interfere with breast cancer progression, we analyzed effects of NAM treatment in MMTV-PyMT mice that already had established spontaneous mammary tumors. Mice received 1% NAM in the drinking water, starting on day 60 when multiple palpable tumors were present in each animal. Controls received water without NAM. Mammary tissue masses were examined on day 80. Importantly, NAD⁺ precursor treatment still significantly inhibited breast cancer progression (Figure 8C and D, and Supplemental Figure 17). The reported weights include all fat pads of each mouse, regardless of tumor presence. Importantly, histopathological analysis revealed that NAM treatment drastically inhibited breast cancer progression (Figure 8C and D, and Supplemental Figure 17). All untreated MMTV-PyMT mice had tumors primarily containing adenoma, early and advanced carcinoma, and little normal and hyperplastic tissue. In contrast, NAM-treated mice had normal mammary fat pads; tumors, if found, were small and consisted predominantly of hyperplastic and adenoma tissue. Furthermore, whereas lung metastases were detected in 4 of 7 untreated animals, no metastases were found in NAM-treated MMTV-PyMT mice (0 of 6), as assessed by comprehensive histology in serial lung sections (data not shown). Importantly, NAM treatment induced autophagy in PyMT mammary tumors in vivo, as shown by p62 degradation in Western blot analyses of individual tumors (Figure 8E and Supplemental Figure 19). We did not detect effects on mTORC1 activity regulation, which suggests that NAM treatment in the drinking water might transiently modulate mTORC1 activity within PyMT mammary tumors in vivo.

**Discussion**

Our present findings establish that the tumor cell NAD⁺/NADH balance controlled by mitochondrial complex I can regulate breast cancer progression. We found that enhancement of complex I activity through expression of NADH dehydrogenase Ndi1 from Saccharomyces cerevisae strongly interfered with tumor growth and metastasis, while inhibition of complex I by knockdown of the subunit NDUFV1 enhanced the metastatic potential of already...
aggressive breast cancer cells. Mutations in complex I were reported in human breast tumors (6, 9–12). mtDNA mutations per se are not known to cause transformation (40), but may contribute to cancer progression, as seen in lung carcinoma with EGFR mutations (41) and in oncocytic tumors with loss of complex I function (14, 42). Furthermore, oncogene activity (e.g., K-Ras transformation) can decrease mitochondrial complex I activity (43), which may support a malignant phenotype (44). Our results revealed a cause-and-effect relationship between complex I function and breast cancer progression.

Enhancement of complex I activity through Ndi1 did not alter tumor cell proliferation in vitro, but clearly reduced tumor growth and metastasis in vivo. Changing access to nutrients and oxygen within the tumor microenvironment influences cell survival and proliferation. Under those conditions, growth regulation through complex I may become critical. We found that this mechanism depended on autophagy. Enhancement of complex I through Ndi1 reduced metastasis and induced p62 elimination, whereas complex I inhibition by NDUFV1 knockdown enhanced metastasis and reduced p62 processing. The critical contribution of autophagy became clear upon ATG5 knockdown, which abrogated the antimetastatic effect of Ndi1 in both breast cancer cell models. Consistent with previous reports (45), we found that basal levels of autophagy facilitated metastasis in MDA-MB-231 cells; this was not seen in MDA-MB-435 cells. Autophagy can inhibit or promote tumorigenesis by supporting tumor cell survival under metabolic stress (27–29). Our results support a dual role of autophagy, while clearly showing that autophagy was required for mitochondrial complex I–mediated reduction of metastatic growth. One of the main regulators of autophagy is mTORC1, which links tumor growth and metabolism (46). We found that mitochondrial complex I modulated mTORC1 and its upstream regulator, AKT, in breast cancer cells, suggesting mTORC1 as a likely pathway through which complex I regulates autophagy.

Our results demonstrated that mitochondrial complex I can control breast cancer progression by regulating the cellular NAD+/NADH balance. Ndi1 expression enhanced this redox balance, particularly under metabolic stress, which indicates its involvement in strong inhibition of tumorigenicity and metastasis through Ndi1. To consolidate a role of complex I controlled NAD+/NADH ratio modulation in breast cancer progression, we experimentally altered tumor cell NAD+/NADH ratios using 2 additional independent approaches. First, we interfered with NAD+ biosynthesis and recycling pathways by knockdown of NAMPT to reduce the NAD+/NADH balance. Second, we enhanced the NAD+/NADH balance by treatment with NAD+ precursors. Importantly, decreasing NAD+/NADH ratios by knockdown of NAMPT, a rate-limiting enzyme in the NAD+ synthesis pathway that converts NAM to NAD+, significantly enhanced metastatic activity in already aggressive human breast cancer cells. NAMPT knockdown enhanced AKT/mTORC1 activities and inhibited autophagy. In apparent opposition, a recent study reported that NAMPT inhibition reduced prostate cancer cell viability (47). NAMPT was therefore suggested as a therapeutic target for inhibition progression of breast cancer (48) and other malignancies (33). We found that the chemical NAMPT inhibitor FK866 induced cell death in vitro and had tumoricidal effects in vivo. In contrast, genetic interference with NAMPT expression in breast cancer cells did not inhibit tumor cell viability and, rather than killing the tumor cells, rendered them more aggressive and metastatic in the animal models. It is possible that FK866 induces cellular stress through alternative mechanisms in addition to depleting NAD+ levels by NAMPT inhibition.

Controversial effects have also been reported for ROS-inducing therapies. While some cancer treatments induce high ROS levels to kill tumor cells, nonlethal increase of ROS may facilitate tumor progression and metastasis (7, 49). A similar concept applies to our present findings, in which NDUFV1 knockdown caused complex I deficiency but did not drastically inhibit OXPHOS. While not affecting cell viability, NDUFV1 knockdown significantly enhanced metastatic activity. Thus, it should be noted that strategies aimed at killing tumor cells by interfering with mitochondrial functions or NAD+ synthesis could, if not effectively lethal, inadvertently produce even more aggressive tumor cell phenotypes. Thus, in the long run, approaches aimed at normalizing mitochondrial functions, particularly complex I activity and NAD+/NADH redox levels, could be therapeutically more effective and safer and would not interfere with normal cell function.

Having established that enhancement of NAD+/NADH levels by augmenting breast cancer cell complex I activity inhibited tumorigenity and metastasis, we used this new concept therapeutically and hypothesized that supplementing tumor cell nutrients with NAD+ precursors, such as NIC or NAM, could interfere with breast cancer progression. NIC and NAM are used to modulate NAD+ levels and showed therapeutic effects in disorders, such as cerebral ischemia, diabetes, and cardiovascular dysfunctions (32, 33). NIC and NAM protect neurons against oxidative damage, whereas subclinical vitamin B3 deficiency is associated with genomic instability and increased cancer risk (50).

We demonstrated that enhancing NAD+ levels through NAD+ precursor treatment effectively initiated experimental metastasis of human breast cancer cells in xenograft models. Importantly, this treatment also inhibited spontaneous metastasis and increased animal survival when the therapy was begun after surgical removal of primary tumors. Furthermore, NAD+ precursor treatment strongly interfered with oncogene-driven breast cancer development and progression in transgenic MMTV-PyMT mice.

Our results provide evidence that the mechanism involves induction of autophagy. NAD+ precursor treatment activated tumor cell autophagy in vitro as well as in vivo and included regulation of mTORC1 activity, as seen in culture. It has been reported that modulation of NAD+/NADH ratios can affect the PI3K/AKT survival pathway through inactivation of PTEN (51). Furthermore, NAD+-dependent sirtuins can modulate mTORC1 through regulation of TSC2 (52) or LKB1 activity (53, 54). Sirtuins may also affect autophagy by controlling FOXO3 degradation (55) or by directly regulating the autophagy proteins ATG5, ATG7, and ATG8 (56). We found that modulation of mTORC1 activity and autophagy by NAM involved SIRT1 activation in MDA-MB-453 cells, and likely depends on additional or alternative mechanisms in MDA-MB-435 and MDA-MB-231 cells. NAD+-dependent sirtuins and PARPs contribute to p53 function and regulate chromatin structure and genomic stability (32, 33, 50), thereby playing emerging roles in tumor progression (57). Moreover, the transcriptional corepressor CstB responds to increased NADH under hypoxia to permit gene expression that promotes tumor cell migration (58). Importantly, BRCA1 tumor suppressor expression is inhibited by low NAD+/NADH ratios (59, 60).

The present study demonstrated that mitochondrial complex I regulation of tumor cell NAD+/NADH levels affects breast cancer growth and metastasis and translated into a new therapeutic
approach for preventing breast cancer progression. This is highly relevant, as the current standard of care for cancer patients relies primarily on chemo- and radiation therapies aimed at killing the tumor cells. Evolutionary models predict that selective pressure imposed by these approaches causes survival of resistant clones that eventually reactivate the disease (61). Based on the central involvement of metabolic tumor cell alterations in cancer, therapeu-
tic normalization of tumor cell metabolism might interfere with the expansion of residual and breakthrough clones. Thus, a combination of standard therapy with NAD⁺ precursor treatment may halt breast cancer progression and prevent relapse.

Methods

Cell culture. MDA-MB-231, MDA-MB-435, MDA-MB-453, and 4T1 cells as well as their variants were stably transduced with Firefly luciferase (F-luc) using lentiviral expression vector eFUW (ubiquitin promoter; provided by B.E. Torbett, The Scripps Research Institute) to analyze tumor growth and metastasis by noninvasive bioluminescence imaging (62). Cells were grown in EMEM supplemented with nonessential amino acids, vitamins, 2 mM l-glutamine, 1 mM pyruvate, and 10% FBS (complete medium). For metabolic stress, cells were cultured in Neurobasal-A Medium (Gibco) supplemented with nonessential amino acids, vitamins, 2 mM l-glutamine, 5 mM or 1 mM glucose, and 2% dialyzed FBS in normoxia (21% oxygen) or hypoxia (<1% oxygen; Modular Incubator Chamber; Billups-Rothenberg). Ndi1 expression. Tumor cells were stably transduced with the Ndi1 gene from S. cerevisiae (19) subcloned into lentiviral expression vector eFUW. Transduced cells were used as pools in all experiments. Ndi1 expression was analyzed by Western analysis, and subcellular localization was analyzed by immunofluorescence, as described previously (18).

NDUV1, NAMPT, and ATG5 knockdown. Lentiviral vectors containing shRNA against NDUV1 (clone TRCN00000025872), ATG5 (clone TRCN00000151963), NAMPT (clone TRCN0000161180), or nontargeting control shRNA (SHC0002) were from Sigma-Aldrich. Knockdown efficiency was quantified by real-time PCR using FastStart Universal SYBR Green Master (Rox) (Roche) and the following primers: ATG5 forward, TCCACTCTTCGAGATGATA; ATG5 reverse, GCCAGCAGGGAATTTTGTTA; NAMPT forward, GCCAGCAGGGAATTTTGTTA; NAMPT reverse, TGATGTGCTGCTTACGATC; NDUV1 forward, AAGTTCTTCGCGATGAGGT; NDUV1 reverse, TGTGAGGATCATG-GCGTAA; GAPDH forward, GGGAAGGTGAAGGTCGGAGT; GAPDH reverse, TCCACTTACAGGATTAAACG. Data were recorded and analyzed using an ABI-PRISM 7700 Sequence Detection System (Applied Biosystems) and Sequence Detector Software (version 2.0, SDS).

Animal experiments. For experimental metastasis, female 6- to 8-week-old CB-17/SCID mice were injected with F-luc-tagged cancer cells: 2.5 × 10⁶ MDA-MB-231 or MDA-MB-435 cells i.v., or 5 × 10⁶ MDA-MB-453 cells into the left cardiac ventricle under stereotactic guidance. For primary tumor growth, 1 × 10⁶ MDA-MB-231 cells or 2.5 × 10⁶ MDA-MB-435 cells were injected into the axillary mammary fat pad. For spontaneous metastasis, female 8-week-old BALB/c mice were injected with 1 × 10⁶ F-luc-tagged 4T1 cells into the fourth mammary fat pad. Tumors were removed surgically when they reached 300 mm³ in size, and animals were euthanized when moribund to determine survival. Mice were imaged weekly (IVIS 200, Xenogen) 10 minutes after i.p. injection of α-luciferin (100 mg/kg). To assess brain metastasis from MDA-MB-453 cells, mice were imaged directly after cardiac injection to validate correct routing by verifying tumor cell signal in the brain region and lack of signal in the lungs. For ex vivo organ imaging at the end of experimental and spontaneous metastasis studies, mice were injected with luciferin 5 minutes before necropsy. For survival analyses, imaging also validated metastasis as the cause of moribundity. Bioluminescence was quantified as photons/s/cm² in defined regions of interest using Living Image software. MMTV-PyMT mouse (strain MT634; gift of W.J. Muller, McGill University, Montreal, Quebec, Canada; ref. 38) were heterozygous for the PyMT transgene and genotyped by PCR using forward primer 5’-CGCGCGAGCGAGAACACTGGAGGAGC-3’ and reverse primer 5’-TCAGAAGCTCGCAGCTCTAGGCG-3’. For NAD⁺ precursor treatment, mice received 1% NIC or NAM in the drinking water, which was changed weekly. For histology, mammary fat pads and lungs were fixed in 10% formalin, sectioned (5 μm), and stained with hematoxylin and eosin (H&E). For the PyMT model, scoring of 4 morphological stages (hyperplasia, adenoma, early carcinoma, and advanced carcinoma; ref. 39) was performed on whole-slide scans (Leica SCN400 Digital Slide Scanner) by image analysis and morphometric measurements (MetaMorph imaging software; version 7.6). Select regions were imaged using a Zeiss Axio Imager M1m microscope and AxioVision software. Lung metastases in MMTV-PyMT mice were quantified on 10 H&E-stained step sections per lung (5 μm thick, 80 μm apart).

Ndi1 integration into cellular respiration. 2 × 10⁶ cells were collected into complete medium, and respiration was measured at 37°C in a 2-ml chamber by high-resolution respirometry using an Oroboros Oxygraph series D and DatLab software (Oroboros Instruments). Routine mitochondrial respiration, corrected for residual oxygen consumption due to oxidative side reactions, was measured in intact cells in complete medium. Functional integration of Ndi1 into tumor cell mitochondrial respiration was determined by oxygen consumption after addition of rotenone (2.5 μM) to inhibit endogenous complex I and final addition of complex III inhibitor antimycin A (2.5 μM) to terminate mitochondrial respiration.

Enzymatic analysis of mitochondrial complex I activity. NADH oxidation to NAD⁺ by complex I was analyzed using an immunocapture complex I enzyme activity assay (MS-141; MitoSciences).

NAD⁺/NADH analysis in whole-cell and mitochondrial extracts. NAD⁺ and NADH were analyzed independently in extracts of whole cells (1 × 10⁶) or isolated mitochondria (from 1 × 10⁶ cells) prepared as described previously (18, 63). Concentrations were determined using a NAD/NADH fluorescence detection kit (Cell Technology Inc.).

mtDNA. mtDNA content was determined by quantitative real-time PCR of total DNA extracted from 2 × 10⁶ cells. PCR reactions were performed in quadruplicates using TaqMan probes specific for MT-RNR1 (mitochondri-

s al RNA) (HS02596859_g1, FAM label) referenced to RPPH1 (RNAse P, VIC label), a nuclear gene. PCR data were recorded and analyzed using the ABI-PRISM 7700 Sequence Detection System (Applied Biosystems) and Sequence Detector Software (version 2.0, SDS).

ATP measurements. ATP was extracted from cell pellets (2 × 10⁶ cells) in triplicates with 100 μl of ice-cold 2M perchloric acid. Extracts were left on ice for 15 minutes, mixed, and then centrifuged at 12,000 g for 5 minutes at 4°C. The supernatant was neutralized with 35 μl of 1M Bicine and 4M potassium carbonate to pH 7.5 and kept on ice for 15 minutes. After centrifugation, ATP concentrations were measured in the supernatant based on ATP-dependent luciferase activity using an ATP determination kit (MitoTechnology). Lactate production. Lactate concentrations were measured in triplicates in conditioned media after culturing 10⁵ cells in 6-well plates (1.5 ml medium/well) for 48 hours. 500 μl medium was deproteinized with 166 μl ice-cold 2M perchloric acid, left on ice for 15 minutes, mixed, and centrifuged at 12,000 g for 10 minutes at 4°C. Supernatants were neutralized with 58 μl of 1M Bicine and 4M potassium carbonate to pH 7 and kept on ice for 20 minutes. After centrifugation, lactate concentrations in the supernatants were determined fluorimetrically at 340 nm excitation and 466 nm emission (460 nm) before and 60 minutes after addition of lactate dehydrogenase, as described previously (5).

Analysis of mitochondrial membrane potential and cell viability. The membrane potential-sensitive fluorochrome tetramethylrhodamine methyl ester (TMRM; Invitrogen) was used to measure mitochondrial membrane poten-
tial. Cells were incubated with 150 mM TMRM for 30 minutes at 37°C in the dark and then analyzed by flow cytometry (FACScalibur). Data from 10,000 events per sample were collected and analyzed by measuring FL-2 fluorescence using CellQuest software. Cell viability was analyzed by measuring the percentage of the nonhypodiploid population (non-sub-G0/G1) by flow cytometry. Cells were fixed in ice-cold 70% ethanol, washed with PBS, and incubated with 50 μg/ml propidium iodide and 100 μg/ml RNase A for 30 minutes at 37°C. Data from 20,000 events per sample were collected by measuring FL-2 fluorescence.

**Immunohistochemistry.** Tumor tissues were paraformaldehyde or zinc fixed, paraffin embedded, sectioned (5 μm), transferred onto glass slides, deparaffinized in safe clear II, and rehydrated in a series of ethanol solutions, p62, Ki67, or Ndi1 staining was performed after antigen retrieval with 1 mM EDTA (pH 8), 10 mM citrate buffer, or 1 mM EDTA plus 10 mM Tris-Cl (pH 8), respectively. Sections were washed 3 times in PBS, treated with 3% H2O2 in PBS (or in methanol for Ndi1) for 15 minutes, blocked in 10% goat serum and 0.3% Triton X-100 in PBS for 1 hour, and incubated with primary antibody against p62 (Santa Cruz Biotechnology Inc.), Ki67 (BD Biosciences — Pharmingen), or Ndi1 (18) overnight (or for 1 hour for p62), followed by incubation with secondary horseradish peroxidase–, alkaline phosphatase–, or biotin-conjugated Abs (Jackson ImmunoResearch Laboratories and Vector Laboratories) for 2 hours. Signal from biotinylated antibodies was amplified using an avidin-biotin complex kit (Vector Laboratories). Horseradish peroxidase was developed with DAB substrate (BD Biosciences — Pharmingen), and nuclei were stained with Contrast Green (Kirkegaard & Perry Laboratories). The slides were washed with isopropanol and briefly incubated in SafeClear II (Fisher Scientific) before mounting in Permount (Fisher Scientific). Images were acquired with a Zeiss Axio Imager M1m microscope equipped with a digital camera, using ×20 air objectives. Digital images were analyzed with AxiosVision 4.6 software (Zeiss).

**Western blot analysis.** Cells were lysed with Laemmli or RIPA extraction buffer. Western blots were incubated with antibodies against Ndi1 (20), p62 (Santa Cruz Biotechnology), phospho-AKT substrates (Ser/Thr), phospho-SC6020-244, phospho-4E-BPThr37/46, LC3B (Cell Signaling Technology), ATG5 (Cell Signaling Technology), β-tubulin (Sigma-Aldrich), NDUF9 (complex I), SDHA (complex II), UQCR1 (complex III), COXI (complex IV), ATP5A1 (complex V), or mitochondrial porin (VDAC1) (Mitosciences). Antibody binding was detected after incubation with secondary antibodies conjugated to IRDye 800, using an Odyssey infrared imaging system (LI-COR Biosciences). Data were analyzed and quantified using Odyssey infrared imaging system application software (version 3.0).

**Statistics.** Statistical comparisons between 2 groups were performed using unpaired 2-tailed Student’s t tests with unequal variance for in vitro results; nonparametric Mann-Whitney tests for in vivo results, since normal distribution could not be assumed; and Kaplan-Meier curves and log-rank test for animal survival. Statistical calculations were performed with GraphPad Prism software. A P value less than 0.05 was considered significant. Results are shown as mean ± SEM of values obtained in independent experiments.

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**Address correspondence to:** Brunhilde Felding-Habermann, Department of Chemical Physiology and Molecular and Experimental Medicine, The Scripps Research Institute, MEM-150, La Jolla, California 92037, USA. Phone: 858.784.2021; Fax: 858.784.2174; E-mail: brunie@scripps.edu.

Byoung B. Seo’s present address is: Department of Animal Resources, College of Life and Environmental Science, Daegu University, Jilhyang, Gyeongsang, Gyeongbuk, Republic of Korea.

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