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Cyclin D1 Repression of NRF-1 Integrates Nuclear DNA Synthesis and Mitochondrial Function

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Abstract

C*yclin D1* promotes nuclear DNA synthesis through phosphorylation and inactivation of the pRb tumor suppressor. Herein, we show that mitochondrial size and activity as measured by Mitotracker were reversed by the expression of cyclin D1 in a Cdk-dependent manner. Nuclear Respiratory Factor 1 (NRF-1), which induces nuclear-encoded mitochondrial genes, was repressed in expression and activity by cyclin D1. Association of NRF-1 with cyclin D1 was demonstrated by co-immunoprecipitation and mammalian two-hybrid assays. It was shown that the cyclin D1-dependent kinase phosphorylates NRF-1 at S47. Cyclin D1 abundance thus coordinates nuclear DNA synthesis and mitochondrial function.

Introduction

Mitochondria function as central components of mammalian cellular survival through production of ATP and re-oxidized NAD. They also govern cell death through mitochondrial membrane-dependent cellular death signals. The mammalian cell contains 10^3-10^4 copies of mitochondrial DNA (mtDNA), 2 ribosomal RNAs (rRNA) and 22 transfer RNAs (tRNA) required for mitochondrial protein synthesis. Nuclear-encoded genes regulate mitochondrial biogenesis and function, respiratory genes, the citric acid cycle and upstream glycotic steps and amino acid metabolism (1). Mitochondrial transcription factor A (mtTFA, also known as Tfam) and nuclear respiratory factors (NRFs) (2, 3) regulate nuclear genes governing mitochondrial function and promoters within the mitochondrial D-loop region, thereby promoting replication and transcription of mtDNA. mtTFA is essential for yeast mtDNA maintenance (4) and *mtTFA*$^{-/-}$ mice have reduced mitochondrial respiratory chain function with depleted mtDNA and oxidative phosphorylation (2).

NRF-1 increases mitochondrial respiratory capacity, induces expression of a subset of genes governing mitochondrial activity in a cell-type specific manner (reviewed in (5)) and enhances mitochondrial responses to the PPARγ co-activator PGC-1 (6). NRF-1-independent mitochondrial gene expression pathways are regulated by PPARs, Sp 1 and other factors. Although mitochondrial gene function is co-dependent upon the nuclear genome and mitochondrial activity alters nuclear gene expression ("retrograde communication" (7)), the mechanism coordinating nuclear DNA synthesis, growth responses and mitochondrial synthesis is unknown.

The *cyclin D1* gene encodes a labile growth factor- and oncogene-inducible component of the holoenzyme that phosphorylates and inactivates the retinoblastoma protein (pRb), promoting nuclear DNA synthesis (8). *Cyclin D1*$^{-/-}$ mouse embryo fibroblasts (MEFs) have a normal cell size and reduced DNA synthesis rates which can be rescued through introduction of cyclin D1 ((9) and data not shown). *Cyclin D1*$^{-/-}$ mice are resistant to oncogene-induced tumors of the skin, gut and mammary gland (10, 11). Herein we examined the possibility that cyclin D1 may also function to regulate mitochondrial activity and thereby couple biosynthetic properties of the nucleus and mitochondria.

Results

To explore the role of cyclin D1 in mitochondrial gene function, we examined the mitochondrial size in *cyclin D1*$^{-/-}$ and littermate wild type control animals. Mitochondrial size was increased 2- to 3-fold in proportion to cellular cytoplasm in hepatocytes (Fig. 1A-D), as well as to the mammary gland adipocyte (Fig. 1E, F) in *cyclin D1*$^{-/-}$ mice. MitoTracker Deep Red 633, used to stain the functioning mitochondria in living cells through a fluorescent signal that is proportional to the mitochondrial activity, showed increased mitochondrial activity in *cyclin D1*-deficient MEFs, hepatocytes, and bone marrow macrophages compared with cyclin D1-expressing cells from littermate control animals (Fig. 1G). Cyclin D1 wild-type and mutant cDNAs were further transduced by retroviral infection into *cyclin D1*$^{-/-}$ MEFs in the MCSV-IRES-GFP vector to allow GFP sorting (Fig. 2A). Mitochondrial function, assessed using MitoTracker (6), was conducted one week after viral transduction. Cyclin D1 inhibited mitochondrial function 52% (Fig. 2A) and also induced DNA synthesis (16% to 52%). Furthermore, the pRb-binding-deficient (GH), and C-terminal deletion (N4) of cyclin D1 also repressed mitochondrial function, whereas the Cdk-binding-defective KE mutant failed to inhibit mitochondrial activity or induce DNA synthesis, indicating the kinase-associated function of cyclin D1 inhibits mitochondrial function.

Examination of the increased mitochondrial size in *cyclin D1*$^{-/-}$ hepatocytes showed the abundance of NRF-1 protein was increased 3-fold in *cyclin D1*$^{-/-}$ compared with wild type liver (Fig. 2B) while the NRF-1 coactivators, P/CAF and PGC-1 were unchanged (data not shown and Fig. 2B). Addition of 10% serum to starved cells

induced cyclin D1 expression, and DNA synthesis, and reduced NRF-1 (Fig. 2C). The NRF-1 target gene reporters, the D-loop reporter and the mtTFA promoter, were both 10-fold more active in *cyclin D1*$^{-/-}$ cells compared with *cyclin D1*$^{+/+}$ cells (Fig. 2D and E), suggesting that cyclin D1 inhibits D-loop transcriptional activity and mtTFA activity. Mutation of the mtTFA promoter NRF-1 or NRF-2, but not Sp1 sites, reduced promoter activity, indicating the enhanced mtTFA promoter activity in *cyclin D1*$^{-/-}$ cells involves increased NRF-1/NRF-2 activity (Fig. 2F). NRF-1 further enhanced while cyclin D1 expression inhibited (without affecting expression of NRF-1), (data not shown) (Fig. 2G) 4xNRE-LUC activity 2- to 3-fold in MCF-7 cells. Cyclin D1 can repress activity of transcription factors, including PPARγ, in a Cdk-independent manner through HDAC recruitment (12). In contrast, cyclin D1 repression of 4xNRE-LUC activity was abrogated by point mutation of the Cdk-binding domain (cyclin D1 KE) (Fig. 2H), but not mutation of the pRb-binding domain (GH), the SRC-1 co-activator binding site (LLAA) or CAK association (T156A) site. The GH mutant induced DNA synthesis suggesting the reduced pRb-binding affinity of this mutant is insufficient to abrogate the DNA-synthesis, promoting function. A T286A mutant, which remains nuclear throughout the cell-cycle in cultured cells (13), repressed NRF-1 activity more than cyclin D1 wt. Expression of the cyclin D1 mutants was similar to wild type in cultured cells (12). NRF-1 transactivation function, assessed with a heterologous DNA-binding domain, was repressed by cyclin D1, but not the KE mutant, again implicating cyclin D1-dependent kinase function in NRF-1 repression (data not shown).

Cyclin D1 associated with NRF-1 *in vivo* Cyclin D1 co-precipitated Cdk4 and NRF-1, but not PGC-1 (12) in immunoprecipitation (IP)-Western blotting of murine liver

extracts (Fig. 3A). FLAG-tagged NRF-1, co-transfected into cells along with cyclin D1, co-precipitated cyclin D1 (Fig. 3B). NRF-1 wt was inserted into the MSCV-IRES-GFP vectors and used to transduce *cyclin D1*$^{-/-}$ and *cyclin D1*$^{+/+}$ MEFs. NRF-1 increased MitoTracker fluorescence 50% more in cyclin D1-deficient cells (Fig. 3C). *In vivo* association of NRF-1 with cyclin D1 was demonstrated by the mammalian two-hybrid system using GAL4-cyclin D1 and VP16-NRF-1 fusion constructs in *cyclin D1*$^{-/-}$ 3T3 cells (Fig. 3D).

Microarray analysis of *cyclin D1*$^{-/-}$ MEFs transduced with a retroviral expression vector for cyclin D1, compared with empty vector identified 210 genes repressed by cyclin D1, 73 of which were similar and 18 identical to the putative NRF-1 site containing gene promoters (Fig. 3E). As NRF-1 induces genes in a cell-type specific manner, we further identified NRF-1-responsive genes in MEFs by transducing cells with a retrovirus expressing NRF-1 and conducting microarray analysis. In these cells mitotracker activity was also induced 3-fold (data not shown). Of the 131 genes induced by NRF-1 (see supplemental material), 22 genes were similar to cyclin D1-repressed genes in MEFs.

As the Cdk4 binding-deficient mutant of cyclin D1 failed to repress NRF-1-mediated transcriptional activity, and since NRF-1 is phosphorylated (14), we compared GST-pRb and GST-NRF-1 as substrates of cyclin D1/Cdk4 kinase. NRF-1 was efficiently phosphorylated by cyclin D1-IP kinase (Fig. 4A). Cyclin D1 co-transfection enhanced ^{32}P-orthophosphate labeled NRF-1 (Fig. 4B, left panel). The p16^{INK4a} peptide inhibits cyclin D1-dependent kinase activity and pRb phosphorylation (15). A p16^{INK4a} peptide (P20), with a D92A substitution lowers the IC_{50} (16) and when linked to the

Antennapedia carrier sequence, inhibit pRb phosphorylation ((16) (and data not shown)) and NRF-1 phosphorylation (Fig. 4B). The $p16^{INK4a}$ peptide (P21) with a A95/96 mutation has an increased IC_{50} for the inhibition of pRB phosphorylation by cyclin-dependent kinases *in vitro* (16). We further showed that it failed to inhibit NRF-1 phosphorylation (Fig. 4B). A comparison was made between NRF-1 and pRb as substrates in cyclin D1-dependent kinase assays. The pRb peptide which contains two sites of phosphorylation incorporated $\gamma^{32}P$ at approximately twice the rate as equal moles of NRF-1 (Fig. 4C). Alignment of the major Cdk4 phosphorylation sites of pRb, p130 and p107 with NRF-1 identified a motif including S47 (Fig. 4D). Point mutation of NRF-1 S47 reduced phosphorylation by cyclin D1-dependent kinase activity 85% (Fig. 4D, right two lanes). In order to determine whether any of the other serine residues within the carboxy terminal fragment of NRF-1, which was sufficient for phosphorylation, could provide rescue function. Analysis was conducted of Alanine insertion mutants of all 12 candidate phosphorylation sites (12xA), with reintroduction of single wild type residues into the cyclin D1 12xA mutant. The proteins were expressed equally by HA-Western blot analysis, but served as poor cyclin D1-dependent kinase activity substrates compared to the HA-tagged wild type NRF-1 (Fig. 4D), indicating S47 is necessary but not sufficient for phosphorylation of NRF-1.

Discussion

The mechanisms integrating cell-cycle progression and/or exit with mitochondrial biogenesis were previously unknown (17, 18). Herein, we demonstrate cyclin D1 ability to repress mitochondrial function and size *in vivo*. This novel function of cyclin D1 represents the first *bone fide,* non-nuclear function for cyclin D1. We further show that the kinase function of cyclin D1 is required for repression of mitochondrial activity and for phosphorylation of NRF-1. Cyclin D1 bound NRF-1 in cells assessed either by immunoprecipitation western blot analysis or by mammalian 2-hybrid. To provide additional corroborative evidence that cyclin D1 inhibited NRF-1-regulated functions, we determined the molecular genetic signature regulated by cyclin D1 and compared this with putative NRF-1 regulated genes based on genome wide location analysis of candidate NRF-1 target genes (19). The genes regulated by cyclin D1, were determined by introducing a cyclin D1 expressing retrovirus into *cyclin D1*$^{-/-}$ MEFs and conducting microarray analysis. A comparison of cyclin D1-repressed genes and genes potentially regulated by NRF-1, based on analysis of NRF-1 binding sites in their promoters, showed substantial overlap (Fig. 3F). We next compared the molecular genetic signature of genes actually regulated by cyclin D1 and those genes actually regulated by NRF-1. The genes regulated by NRF-1 were determined by transducing MEFs with an NRF-1 expressing retrovirus and conducting microarray analysis. Of the 254 candidate NRF-1-inducible genes and 210 genes repressed by cyclin D1, 73 similar genes and 18 identical genes were shared between the two gene sets.

We examined the physiological regulation of cyclin D1 and NRF-1 expression. NRF-1 was increased 3-fold in cyclin D1 null cells. Cyclin D1 levels and NRF-1 expression were inversely correlated during cell cycle progression, with serum-induced cyclin D1 expression peaking at 12 hours and NRF-1 expression decreasing in 8-12 hours. The reduction in NRF-1 abundance at 8 hrs, shortly after cyclin D1 levels increased Fig. 2C), was consistent with our model in which cyclin D1 inhibits NRF-1 function (Fig. 5). Together these studies identified, for the first time, the genes regulated by NRF-1 and those NRF-1 regulated genes that showed significant overlap with cyclin D1-regulated genes. The prior studies of NRF-1 regulation by serum, in other cell types showed no change in levels or activity of cytochrome oxidase and several NRF-1 targets within 12 hrs of serum stimulation (20). ChIP assays in *Cam et al*. show no change in NRF-1 binding to NRF-1 sites during serum treatment. At this time, no NRF-1 regulatory serum-inducible kinase has been isolated and multiple phosphorylation sites do exist in NRF-1, likely regulated by distinct kinases. The finding herein that NRF-1 is inactivated by cyclin D1, and phosphorylated by cyclin D1-dependent kinase does not preclude the possibility that NRF-1 may be regulated by other serum-regulated kinases, as many proteins are under regulation by distinct kinases regulating proliferative and anti-proliferative signals. It remains to be determined whether other cell-cycle-regulatory kinases phosphorylate and repress NRF-1.

Cyclin D1 repressed NRF-1 induced genes by microarray analysis. Additionally, cyclin D1 repressed mitochondrial genes that were not considered direct NRF-1 targets as defined by either the presence of putative NRF-1 sites in their promoters or by microarray analysis in MEFs. Cyclin D1 expression repressed mtTFA abundance and promoter

activity requiring, primarily, the NRF-1 and NRF-2 binding sites. mtTFA binds to sequences in the divergent heavy and light-chain promoters of the mitochondrial D-loop region where it stimulates transcription from mitochondrial DNA templates. Consistent with the finding that cyclin D1 repressed NRF-1 and mtTFA, cyclin D1 inhibited D-loop transcriptional activity. mtTFA promotes differentiation in myoblasts (21-23) while cyclin D1 inhibits differentiation of myocytes (24) and adipocytes (12). Cyclin D1 antagonizes NRF-1 and mitochondrial function while inducing DNA synthesis, thereby potentially coupling biosynthetic priorities within the cell.

In this study we proposed that cyclin D1 may integrate cell-cycle progression and/or exit with mitochondrial biogenesis (Fig 5). We do in fact demonstrate the increased abundance of cyclin D1 led to reduced mitochondrial activity. The outcome of reduced mitochondrial activity would be anticipated in order to shift glucose metabolism toward cytosolic glycolysis. Whether such a triage of substrate utilization or metabolic prioritization occurs in the presence of increased cyclin D1 abundance remains to be determined. A shift towards cytosolic glycolysis is known to occur during tumor progression and is a component of the metabolic change described by Dr. O. Warberg in 1930. Known components of the mitochondria-to-nucleus retrograde pathway include CREB, mRpL12, and aconitase, (which produces α-ketoglutarate) (25-27). In *Drosophila*, in which proliferation rates are partially uncoupled from growth, cyclin D1 induces cell size and Hif-1 prolyl hydroxylase (HPH) activity (25). But in mammalian cells, HPH is not required for proliferation by cyclin D1/Cdk4, mitochondria inhibit HPH hydroxylase and cyclin D1 does not affect cell size (28, 29). Mitochondria produce ATP (30), regulate single carbon metabolism, fatty acid metabolism and oxidative glycolysis

(5) and are a potential target for cancer therapies (31). As cyclin D1 is inhibited by differentiation and induced by oncogenes, this new function of cyclin D1 may provide a mechanism by which select oncogenes and growth factors contribute to tumor maintenance.

Materials and Methods:

Mice

Mice homozygously deleted of the *cyclin D1* gene (*cyclin D1*$^{-/-}$) (32) on a mixed C57Bl/6x129/SvJ background were maintained as described previously (9). Genotyping was performed on tail genomic DNA by PCR under the following conditions: denaturing at 96°C for 1 min, followed by 30 cycles of 94°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 30 sec with a final extension at 72° for 5 min. The cyclin D1 specific primers were described (32).

Cell Culture

Day 5 Bone marrow macrophage (BMM) from *cyclin D1*$^{+/+}$ and *cyclin D1*$^{-/-}$ mice were prepared as previously described (33) and cultured in supplemented α-modified minimal essential medium (α+MEM) (Life Technologies, Gaithersburg, MD.) containing 15% fetal bovine serum (FBS) (Life Technologies) and 120 ng/ml human recombinant CSF-1 (gift of Chiron Corp., Emeryville, CA). MCF-7 and HEK 293T cells were maintained in DMEM containing penicillin and streptomycin (100 mg of each/liter) and supplemented with 10% FBS.

Primary MEFs were isolated following protocol described in (34) from day 14 post coitus (d.p.c.) mouse embryos. Briefly, embryos were separated and minced and then incubated in a solution with 0.05% trypsin and 1 mM EDTA at 37°C. The supernatant was collected by centrifugation at 1,000g for 3 minutes.

Plasmids, transfections and reporter assays

The expression vectors pCMV-cyclin D1, CMV-cyclin D1-KE, CMV-cyclin D1 GH, pCMV-cyclin E, pCMV-cyclin A and RSV- and CMV-*Renilla* luciferase reporter were

previously described (35). The human cyclin D1 mutants were derived by PCR-directed amplification using sequence-specific primers and cloned into pRC/CMV. The reporter plasmids NRF_4-Luc (containing 4 tandem NRF-1 sites) (36), mtTFA-RC4wt/PGL3 (37), reporters with NRF-1, NRF-2, or Sp1 mutations (6) and the expression vector pSG5 NRF-1 (38) were previously described. Wild type and mutant cyclin D1 and NRF-1 cDNA fragments were inserted into the EcoRI site of the pMSCV-IRES-GFP vector to make pMSCV-IRES-GFP-cyclin D1 and pMSCV-IRES-GFP-NRF-1.

Mammalian two-hybrid was performed following manufacturer's instructions (Promega). Cyclin D1 cDNA was cloned into pBind vector and NRF-1 cDNA was cloned into pACT vector to generate fusion proteins with the DNA binding domain of GAL4 (Gal4-cyclin D1) and the activation domain of VP16 (VP16-NRF-1), respectively. The pG5Luc vector contains five GAL4 binding sites upstream of a minimal TATA box, which in turn, is upstream of the firefly luciferase gene.

DNA transfection and luciferase assays were performed as previously described (39). Cells were transfected by Superfect Transfection reagent (Qiagen, Valencia, Ca). The medium was changed after 5 h, and luciferase activity was determined after 24 h. At least two different plasmid preparations of each construct were used. In cotransfection experiments, a dose-response curve was determined in each experiment with 20 ng of expression vector and the promoter reporter plasmids (1 µg). Luciferase activity was normalized for transfection with *Renilla* luciferase reporter (Promega) as an internal control. Luciferase assays were performed at room temperature with an Autolumat LB 953 (EG&G Berthold). The -fold effect was determined by comparison to the empty

expression vector cassette, and statistical analyses was performed using the Mann Whitney *U*-test.

Cytochemistry and electron microscopy

Liver and mammary gland from cyclin D1 wt and cyclin $D1^{-/-}$ mice were removed under anesthesia. Slices (approximately 2 mm thick) were prepared by hand cutting with a razor and were immediately placed into cold fixative consisting of a mixture of 4 % paraformaldehyde and 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4 for 3hrs with continuous skaking. Non-frozen sections (~20 μm) of the aldehyde fixed tissue slices were prepared using a Lancer vibratome sectioning instrument (Polysciences, Warrington PA). Sections were tested for catalase activity using an incubating medium containing 3, 3" diaminobenzidine tetrahydrochloride (Sigma) as a substrate at pH 9.7 to visualize peroxisomes and microperoxisomes. The incubated sections were then fixed in 1% osmium tetraoxide (Polysciences), alcohol dehydrated and embedded in Epon. Ultrathin sections (approximately 500 angstroms) were prepared using a LKB ultramicrotome (LKB, Sweden), stained with lead citrate and examined with a Philips 300 electron microscope (40). All pictures were taken at the same magnification, i.e at 5000X. To determine mitochondrial size and number, stereology was performed according to the method of Weibel (12). A grid of approximately 1cm squared was placed over each print photo (magnificatoin of 13,500X) and mitochondria within the grid were counted.

FACS analysis

Bone marrow macrophages, hepatocytes, and MEFs derived from *cyclin* $D1^{+/+}$ and *cyclin* $D1^{-/-}$ mice were incubated in medium containing 50 nM of MitoTracker Deep Red 633

(Molecular Probes) for 30 min at 37°C. Cells were then trypsinized and resuspened in PBS. The MitoTracker fluorescence of these positive cells was analyzed by flow cytometry.

Western Blot

The antibodies used in Western blot analysis were cyclin D1 antibody Ab3 (NeoMarkers Lab Vision Corporation, Fremont, CA), cyclin D1 antibody 72-13G, PGC-1 (H-300) (Santa Cruz) and NRF-1 (Felorence). Proteins were visualized by the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ). The abundance of immunoreactive protein was quantified using a densitometer (Image Quant version 1.11, Molecular Dynamics Computing Densitometer, Sunnyvale, CA).

Retroviral infection and FACS sorting

Retroviruses were prepared as described previously (32). Briefly, pMSCV-IRES-GFP was co-transfected with an ecotropic, replication-defective helper virus pSV-ψ-E-MLV into 293T cells, by calcium phosphate precipitation. The retroviral supernatants were harvested 48 h after transfection and filtered through a 0.45-μm filter. *cyclin D1*$^{-/-}$ MEFs were incubated with fresh retroviral supernatants and 8 μg/ml polybrene, centrifuged for 2 hours at 1200 rpm at 25°C and incubated at 37°C overnight. Following 2 days of culture in DMEM with 10% FBS, GFP positive cells were sorted by FACStar (Beckton Dickinson).

High Density Array Expression Analysis

Total RNA was isolated from retrovirus vector infected MEFs (infected with either MSCV- IRES-GFP vector or MSCV-IRES-FLAG-NRF-1) using Trizol. Total RNA was used to probe an Affymetrix GeneChip® Mouse Expression Set 430 array (Affymetrix,

Santa Clara, CA). RNA quality was determined by gel electrophoresis. Probe synthesis and hybridization was performed according to the manufacturer's protocol (see eukaryotic target preparation section at http://www.affymetrix.com/support). Three arrays were used for each condition. Analysis of the arrays was performed as described (41).

In vivo labeling

293T cells were plated 24 h before transfection. Prior to transfection, cells were washed incubated with phosphate-free DMEM medium for 2 h, and then ^{32}P-orthophosphate was added to the cell culture medium at 1 mCi/ml. Lysates were prepared and precipitated with M2 antibody for at least 6 h. Beads were washed with 6 changes of lysis buffer and protein-bound beads were denatured in SDS buffer. Protein was resolved by SDS-PAGE electrophoresis. Gels were dried and subjected to autoradiography.

Figure Legends:

Figure 1. *Cyclin D1*-deficiency enhances mitochondrial size and function. (A, B). Transmission electron microscopic (TEM) images of hepatocytes from liver tissue of *cyclin D1*$^{+/+}$, left in (A) and (B, red box, enlarged area) and *cyclin D1*$^{-/-}$ right images in (A) and (B, yellow box, enlarged area) show increased mitochondria size in *cyclin D1*$^{-/-}$ mice. Catalase-positive peroxisomes (dark spherical structures) are evident in (A). Magnification: (A) original, 5000X. (C, D). Mitochondrial size is increased in proportion to cellular cytoplasm shown through stereoscopy in *cyclin D1*$^{-/-}$ and in *cyclin D1*$^{+/+}$ hepatocytes. (E) TEM of mammary gland adipocytes with enlarged view (F), reveals increased mitochondria size. (G). Mitochondrial activity in hepatocytes (Ga), Bone Marrow Macrophages (Gb) and MEFs (Gc) derived from either *cyclin D1*$^{+/+}$ or *cyclin D1*$^{-/-}$ mice assessed using MitoTracker (Deep Red, 50 nM).

Figure 2. NRF-1 activity is enhanced in *cyclin D1*-deficient cells. (A) Vectors encoding cyclin D1 Wt and mutants in the vector MSCV-IRES-GFP were used to transduce *cyclin D1*$^{-/-}$ MEFs for a week. Cells were stained with Mitotracker Red CMX Ros for 30 min. The GFP positive cells were sorted and MitoTracker fluorescence and cell cycle was analyzed by flow cytometry. The cyclin D1 K112 residue was required for inhibition of Mitochondrial activity and induction of DNA synthesis. (B, C). MCF-7 cells were starved in DMEM supplemented with 0.2% FBS for 48 hours. Cells were harvested at time points as indicated. Total cell lysates were prepared and subjected to Western blotting analysis to detect cyclin D1 and NRF-1 expression. β-tubulin and GDI were included as loading controls. (D, E) *Cyclin D1*$^{+/+}$ and *cyclin D1*$^{-/-}$ 3T3 cells were transfected with vectors in which the reporter activity was driven by the various

promoters driving luciferase reporter genes for wild type mtTFA, D-loop, or CMV or (F) mtTFA promoter reporter plasmids encoding mutant response elements for NRF-1, Sp-1, or NRF-2. The luciferase activity is shown as the mean ± SEM (N≥6). (G) MCF-7 cells were transfected in 24-well plates with 1 μg of a luciferase reporter gene containing four copies of NRF-1 response elements, 0.5 μg of pSG5 vector or pSG5-NRF-1 expression plasmid and the cyclin D1 expression vector. Luciferase activity was normalized to a co-transfected pRL-TK Luc control. (H) Mutant cyclin D1 expression plasmids were compared for NRF-1 repression function. (mean ± SEM, N>3 separate experiments, each performed in quadruplicate).

Figure 3. Cyclin D1 interacts with NRF-1. (A) Lysates from mouse liver were subjected to cyclin D1 antibody immunoprecipitation (IP). IP products were resolved on SDS-PAGE gel with Western blot to NRF-1 or PGC-1. Cyclin D1 associates with NRF-1, but not PGC-1 *in vivo*. (B) 293T cells were transfected with FLAG-tagged NRF-1 and cyclin D1. Cell lysates were subjected to IP with either IgG or anti-FLAG antibody. IP products were resolved on SDS-PAGE gel followed by Western blot with either FLAG, for NRF-1, or cyclin D1 antibodies. (C) Vectors encoding NRF-1 (in the vector MSCV-IRES-GFP) were used to transduce *cyclin D1*$^{-/-}$ and *cyclin D1*$^{+/+}$ MEFs. Cells were stained with MitoTracker Red CMX Ros for 30 min. The GFP positive cells were sorted and MitoTracker fluorescence analyzed by flow cytometry. (D) The Gal4-cyclin D1 and VP16-NRF-1 fusion constructs were transfected with the pG5luc reporter into *cyclin D1*$^{-/-}$ 3T3 cells. Firefly luciferase activity was quantitated using the Dual-Luciferase® Reporter Assay System (Promega). Interaction between these two proteins (lane 7) increased luciferase activity compared to negative controls. (E) Comparison of cyclin D1-regulated

genes as determined by microarray analysis with genome wide location analysis of candidate NRF-1 target genes comparing genes found on the HU13K and MGU74 chips (http://www.jci.tju.edu/pestell/papers/gfp_vs_d1.xls and http://www.jci.tju.edu/pestell/papers/gfp_vs_nrf1.xls) (19).

Figure 4. NRF-1 serves as substrate of cyclin D1-dependent kinase. (A) GST-NRF-1 was incubated with an immunoprecipitated cyclin D1/Cdk4 kinase complex in the presence of ^{32}p-γ-ATP. Left panel: coomassie blue staining of input GST protein; Right panel: γ^{32}p incorporation into GST-NRF-1. GST and GST-Rb were the negative and positive controls for kinase activity. (B) Left panel: 293T cells were transfected with FLAG-NRF-1 and cyclin D1 or control empty vector. Right panel: 293T cells transfected with FLAG-NRF-1 and cyclin D1 were treated with p16^{INK4a} peptide (20 μM) corresponding to amino acids 84-103 of the human p16^{INK4a} protein (DAAREGFLATLVVLHRAGAR), with a C-terminal 16 amino acids Penetratin (RQIKIWFQNRRMKWKK) or control peptide. (Bio-synthesis, Inc. Lewisville, TX), (42). NRF-1 phosphorylation was abrogated by the p16^{INK4a} peptide. Cells were pulse-labeled with γ^{32}P-orthophosphate. NRF-1 protein was precipitated with anti-FLAG M2 antibody and subjected to autoradiography. (C) Equal amounts of either the GST-NRF-1 N70 fusion protein or GST protein were incubated with 200 ng of purified cyclin D1/Cdk4 complex and [γ^{32}P]-ATP (left panel). In the autoradiogram the arrows indicate the phosphorylated fusion protein. GST-Rb serves as a positive control. The phosphorylated bands were quantified by densitometry scanning (right panel). (D) Alignment of Cdk4 phosphorylation sites for pRb, p107, p130 and NRF-1. 293T cells were transfected with expression vectors encoding HA or FLAG-tagged NRF-1 and

mutants along with cyclin D1-NRF-1 mutants included one in which a single potential phosphorylation site was restored in all sites (12xA) or one with a single point mutant of S47. NRF-1 and mutant proteins were precipitated with anti-HA antibody and subjected to autoradiography.

Figure 5. Schematic representation of the mechanism by which cyclin D1 inhibits mitochondrial function. Cyclin D1-dependent kinase phosphorylates and inhibits NRF-1 and thereby mtTFA and mitochondrial activity. Additional NRF-1-independent mechanisms regulating mitochondrial activity remain to be defined.

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Supplemental material. Tree view display of microarray analysis and Mitotracker analysis of MEFs transduced with NRF-1 retrovirus.