Activation of Akt Is Essential for the Propagation of Mitochondrial Respiratory Stress Signaling and Activation of the Transcriptional Coactivator Heterogeneous Ribonucleoprotein A2

Manti Guha,* Ji-Kang Fang,* Robert Monks,† Morris J. Birnbaum,† and Narayan G. Avadhani*  

*Department of Animal Biology and the Mari Lowe Center for Comparative Oncology, School of Veterinary Medicine; and †Institute for Diabetes, Obesity and Metabolism, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104

Submitted March 9, 2010; Revised July 7, 2010; Accepted August 11, 2010  
Monitoring Editor: Thomas D. Fox  

Mitochondrial respiratory stress (also called mitochondrial retrograde signaling) activates a Ca\(^{2+}\)/calcineurin-mediated signal that culminates in transcription activation/repression of a large number of nuclear genes. This signal is propagated through activation of the regulatory proteins NFkB c-Rel/p50, C/EBP\(\beta\), CREB, and NFAT. Additionally, the heterogeneous ribonucleoprotein A2 (hnRNP\(\text{A2}\) functions as a coactivator in up-regulating the transcription of Cathepsin L, RyR1, and Glut-4, the target genes of stress signaling. Activation of IGF1R, which causes a metabolic switch to glycolysis, cell invasiveness, and resistance to apoptosis, is a phenotypic hallmark of C2C12 myoblasts subjected to mitochondrial stress. In this study, we report that mitochondrial stress leads to increased expression, activation, and nuclear localization of Akt1. Mitochondrial respiratory stress also activates Akt1-gene expression, which involves hnRNP\(\text{A2}\) as a coactivator, indicating a complex interdependency of these two factors. Using Akt1\(^{−/−}\) mouse embryonic fibroblasts and Akt1 mRNA-silenced C2C12 cells, we show that Akt1-mediated phosphorylation is crucial for the activation and recruitment of hnRNP\(\text{A2}\) to the enhanceosome complex. Akt1 mRNA silencing in mtDNA-depleted cells resulted in reversal of the invasive phenotype, accompanied by sensitivity to apoptotic stimuli. These results show that Akt1 is an important regulator of the nuclear transcriptional response to mitochondrial stress.

INTRODUCTION

Mitochondria play critical roles in respiration-coupled energy production, amino acid and fatty acid metabolism, Ca\(^{2+}\) homeostasis, heme biosynthesis, Fe\(^{2+}\) homeostasis, and the integration of apoptotic signals (Babcock et al., 1997; Kroemer et al., 1998; Loeb et al., 2005; Taylor and Turnbull, 2005). Mitochondrial dysfunction is associated with various human diseases, from tissue-specific conditions to generalized whole-body disorders, including cancer (Taylor and Turnbull, 2005). Proliferating tumors in human patients and animal models have been shown to contain mutated or deleted mitochondrial DNA, and/or dysfunctional mitochondria. Mutations in mtDNA and reduced mtDNA copy number have been reported for numerous tumors (Horton et al., 1996; Parrell et al., 2001; Okochi et al., 2002; Mazurek and Eigenbrodt, 2003; Lee et al., 2004; Linnartz et al., 2004; Meierhofer et al., 2004; Biswas et al., 2005a; Lievre et al., 2005; Petrovs et al., 2005; Taylor and Turnbull, 2005; Shidara et al., 2005b; Kulawiec et al., 2009; Singh et al., 2009). A cybrid cell line carrying mutations in the mtDNA-encoded ATPase 6 gene exhibited increased tumorigenicity and reduced apoptosis, indicating a direct role for mtDNA mutations in tumor progression (Petros et al., 2005; Shidara et al., 2005a). Similarly, heteroplasmic but not homoplasmic mutations in mtDNA encoded ND5 gene in human 143B osteosarcoma cells were shown to be tumorigenic (Park et al., 2009). We reported earlier that partial mtDNA depletion in C2C12 skeletal myoblasts and A549 lung carcinoma cells results in highly invasive phenotypes that are resistant to chemically induced apoptosis (Amuthan et al., 2002; Biswas et al., 2005a). Induced proliferation and tumor growth promotion by mitochondrial stress signaling in immortalized C2C12 myoblasts is of special significance for cancer promotion (Amuthan et al., 2001; Biswas et al., 2005a; Guha et al., 2007). Others have shown that mtDNA depletion induces resistance to apoptosis in 143B osteosarcoma (Dey and Moraes, 2000), T47D breast carcinoma (Yu et al., 2009), SK-Hep1 (Kim et al., 2002), and prostate cancer cells (Moro et al., 2009). Depletion of mtDNA in LNCaP androgen-dependent pros-
Role of Akt in Mitochondrial Stress

**Materials and Methods**

*Murine C2C12 skeletal myoblasts (ATCC, Manassas, VA; CRL1772) were grown in DMEM (Life Technology, Carlsbad, CA) supplemented with 10% fetal bovine serum and 0.1% gentamicin. Depletion of mtDNA was carried out by ethidium bromide treatment (EtBr, 100 ng/ml for 30 passages) as described before (Biswas et al., 1999). Selected clones containing ∼20% mtDNA were grown in the presence of 1 mM sodium pyruvate and 30 μg/ml uridine. We also used 2,3-dideoxycuridine (ddC; 10 μM, 120 h) in some experiments (as mentioned) for depleting the mtDNA. Reverted cells represent mtDNA-depleted cells (85% depletion) grown subsequently for 30 cycles in the absence of EtBr, for reversing the mtDNA content to 80% of control cells. The mtDNA content was estimated by real-time PCR using a probe for mouse mtDNA (D-loop) and normalized using the nuclear Ldβ, a single-copy gene. The sequences of the primers used are as follows: mtDNA: ACTATCCCTTTCCCACTTTC (forward) and TGGTGCTACGGGCTGATTA (reverse); and nuclear gene Ldβ: AGCTGTGTGTCGGCTACAGT (forward) and ATCCCTTTCCCACTTTC (reverse). Aliquots from the same parent stock cultures of control, depleted, and reverted cells were used in all experiments. The relative mtDNA content of each cell group is shown in Figure S1.*

**Preparation of Nuclear and Cytosolic Protein Extracts**

Nuclear and cytosolic protein fractions were isolated from different cells as described previously (Biswas et al., 1999; Guha et al., 2007).

**Immunoblot Analysis**

Protein content was measured by the method of Lowry (Lowry et al., 1951). Total cell lysates, cytosolic fractions, and nuclear fractions (30 μg) were solubilized in Laemmli’s sample buffer, resolved by electrophoresis on 10% or 12% polyacrylamide gels, and subjected to immunoblot analysis. The immunoblots were developed using the Super Signal West Femto maximum sensitivity substrate from Pierce Chemical Co. (Rockford, IL). Antibodies to Akt1/2, pAkt (Ser473), and pAkt (Thr308) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies to p-Serine, p-Thr, and p-Tyr were from Sigma Chemicals (St. Louis, MO). Antibodies to nuclear p97 was from Affinity Bioreagents (Golden, CO). Antibodies to DHFR (dihydrofolate reductase), β-actin, and hnRNPA2 were from Santa Cruz Biotechnology (Santa Cruz, CA).

**CnAa, IGF1R, and Akt1 mRNA Knockdown by Small Interfering RNA**

Pre-designed small interfering RNAs (siRNAs) for mouse CnAa (ID no. 292199), IGF1R (ID no. 159115), and Akt1 (ID no.162426), and negative controls (scrambled siRNA) were purchased from Ambion (Austin, TX). Control and mtDNA-depleted cells (10² cells/well) were transfected with pre-annexed double-stranded siRNAs (50 μM stock) at a final concentration of 30 nM by the method of reverse transfection. Transient transfections were carried out in triplicate using siPORT NeoFX reagent (Ambion). RNA was isolated 48 h after transfection using Trizol reagent (Invitrogen, Carlsbad, CA), and the level of silencing of CnAa, IGF1R, and Akt1 mRNA was quantified by real-time PCR, as described later in this section.

**Generation of Stable hnRNPA2-silenced Cell Lines**

hnRNPA2-silenced stable knockdown of C2C12 cells were described before (Guha et al., 2009). Stable cell lines were generated after transfection (Fugene 6 reagent; Roche Molecular Biochemicals, Indianapolis, IN) of mtDNA-depleted C2C12 cells with hnRNPA2si3-pSilencer2.1neo (or pSilencer2.1neo vector alone) containing a neomycin resistance gene. HnRNPA2-silenced cell lines were generated using G418 (1 mg/ml) as a selection marker.

**Cloning and Transfections**

Human Akt1 promoter (~900 to +1), a kind gift from J. Q. Chen, was subcloned into the pGL3 mammalian expression vector (Promega, Madison WI). The c-Rel, C/EBP, NFAT, and CREB genes were cloned into the pCMV4 expression vector. hnRNPA2 cDNA was subcloned into pCI for transfections of C2C12 cells. The WT, T98A, and S219A mutant hnRNPA2 constructs in pCDNA 3.1 (+) hygro plasmid were generated by Gene Pass (Nashville, TN). Transfections were carried out using Fugene 6 reagent, following the manufacturer’s suggested protocol (Promega). The promoter DNA constructs were cloned in the pGL3 vector (1 μg) and 0.5 μg of a renilla luciferase construct (Promega) were used as an internal control. Luciferase activity was assayed using the Dual-Luciferase reporter assay system from Promega. Transfections with various cDNAs were carried out using 0.2 μg of cDNA constructs.

**mRNA Quantitation by Real-Time PCR**

Total RNA was isolated using Trizol reagent as per supplier’s protocol (Invitrogen). cDNA was generated from 5 μg RNA using the cDNA Archive kit from Applied Biosystems (Foster City, CA), and 50 ng of this cDNA was...
used as a template for each reaction. Relative quantification of CnA, IGF1R, Akt1, and Akt2 mRNA by real-time PCR was done using SYBR Green (Applied Biosystems) in an ABI Prism 7300 sequence detection system (Applied Biosystems). Levels of mRNA were normalized to β-actin as an endogenous control.

**Immunoprecipitation**

To ensure nearly equal amounts of input Akt levels in the control and mtDNA-depleted cell extracts, we used 100 μg of protein from mtDNA-depleted cells and 300 μg protein from control cells. The nuclear and cytosolic fractions were immunoprecipitated overnight at 4°C with hnRNPA2 antibody (20 μg/ml). The immune complexes were collected onto protein A–agarose beads (Sigma Chemicals) and washed extensively. The immunoprecipitates were extracted from the beads with 2× Laemmli buffer devoid of β-mercaptoethanol at 95°C for 5 min for further analysis.

**Akt Activity**

Akt activity was measured using an Akt/protein kinase B (PKB) kinase activity assay kit (Stressgen Bioreagents, Victoria, BC, Canada). This assay is based on an ELISA that utilizes a synthetic peptide as a substrate for PKB and a polyclonal antibody that recognizes the phosphorylated form of the substrate.

**Annexin V Assay**

The assay for cells undergoing early apoptosis was performed using the Guava Nexin Kit (Guava Technologies, Hayward, CA) according to the manufacturer’s suggested protocol. This method utilizes annexin V-PE to detect phosphatidylserine on the plasma membrane as a marker of apoptotic cells. Cells (10⁶ each) were transfected in triplicate with Akt1 and mock siRNAs, harvested after 48 h, washed with 1 ml H11003/H9262 depleted cells and 300 μl Guava Nexin Buffer (Guava Technologies), and resuspended in the same buffer. After labeling with Annexin V-PE, the percentage of apoptotic cells was quantified using the Guava Cytometer (Guava Technologies), and an equal number of viable cells (1 × 10⁴) were seeded on top of the Matrigel layer. After incubation for 24 h at 37°C, noninvading cells in the Matrigel layer were quantitatively removed, and the microporous membrane containing invaded cells was stained and viewed under an Olympus BX 61 fluorescence microscope (Melville, NY) as described previously (Amuthan et al., 2001; Guha et al., 2007). Each experimental set was carried out in triplicate, and at least six fields were examined within any single experiment for each condition.

**In Vitro Invasion Assay**

The in vitro invasion assays were carried out as described previously (Amuthan et al., 2001). The Matrigel invasion chambers were prepared at 1:2 dilution of Matrigel (Becton Dickinson, Belford, MA) as described previously (Guha et al., 2007). Cells from three wells counted in a Guava Personal Cytometer (Guava Technologies) and an equal number of viable cells (1 × 10⁴) were seeded on top of the Matrigel layer. To ensure nearly equal amounts of input Akt levels in the control and mtDNA-depleted cell extracts, we used 100 μg of protein from mtDNA-depleted cells and 300 μg protein from control cells. The nuclear and cytosolic fractions were immunoprecipitated overnight at 4°C with hnRNPA2 antibody (20 μg/ml). The immune complexes were collected onto protein A–agarose beads (Sigma Chemicals) and washed extensively. The immunoprecipitates were extracted from the beads with 2× Laemmli buffer devoid of β-mercaptoethanol at 95°C for 5 min for further analysis.

**In Vitro Kinase Assay**

The in vitro kinase assay was carried out according to the methods of Summers and coworkers (Summers and Birnbaum, 1997). 0.1 μg of Recombinant Akt (Millipore, Billerica, MA) was incubated for 30 min at 37°C with purified His-hnRNPA2 (1.0 μg) in a buffer containing 10 mM HEPES (pH 7.4), 1 mM MgCl₂, and 1 mM MnCl₂ in the presence of 10 μCi [γ-³²P]ATP.

**Chromatin Immunoprecipitation Analysis**

Chromatin immunoprecipitation (ChIP) assays were performed following the protocol of Upstate Biotechnologies (Lake Placid, NY). Cells were fixed by adding 1% formaldehyde to the culture medium and were incubated for 10 min at 37°C. Cells were washed in 1× PBS (containing protease inhibitors), scraped, and pelleted at 2000 × g for 5 min at 4°C. The cell pellet was suspended in SDS lysis buffer (containing 1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1) and incubated for 10 min on ice. The cross-linked cell lysates were sonicated using an Ultrasonic processor sonicator (10-s pulses, 20 times, on ice), and the size of sheared DNA was checked on 0.8% agarose gel, such that the DNA fragments were 200-1000 base pairs. The samples were centrifuged for 10 min at 15,000 × g, 4°C. The supernatant was diluted sixfold in a buffer (pH 8.1) containing 167 mM NaCl, 16.7 mM Tris-HCl, 1.2 mM EDTA, 0.01% N,N,N′,N′-tetramethyl-ethylenediamine, 1 mM MgCl₂, and 1 mM MnCl₂ (Applied Biosystems). Levels of mRNA were normalized to β-actin as an endog- enous control.

**V. Guha et al.**

In Figure 1, increased Akt1 protein and mRNA levels in cells subjected to mitochondrial respiratory stress, either by mtDNA depletion or treatment with CCCP. (A) Top, immunoblot analysis of total cell lysate (30 μg protein each) from control, mtDNA-depleted C2C12 cells, and control C2C12 cells treated with CCCP (25 μM, 2 h) using Akt1 antibody (1:1000 dilution). Bottom, immunoblot analysis of total cell lysates (30 μg each) from control, mtDNA-depleted, and reverted C2C12 cells using Akt1 antibody. β-Actin was used as an internal loading control. (B) Real-time PCR analysis of mouse Akt1 and Akt2 mRNA levels in control, mtDNA-depleted, and reverted C2C12 cells and also in control C2C12 cells treated with CCCP (25 μM, 2 h). (C) Real-time PCR analysis of Akt1 and Akt2 mRNA in mouse embryonic fibroblasts (MEFs) treated without and with CCCP (25 μM, 2 h). (D) Immunoblot analysis of total cell extracts (30 μg each) of control and mtDNA-depleted A549 lung carcinoma cells with antibody to Akt and phospho-Ser473 Akt. (E–G) Immunoblot analysis of nuclear extracts from H9C2 cells treated with CCCP, mtDNA-depleted RAW 264.7 cells, and CCCP treated MCF-7 cells with Akt and Ser437 P Akt antibodies. Although not shown, mtDNA contents of A549 and MCF-7 cells were ~20–25% of control cells. In B and C, mean ± SEM values were derived from three to four separate experiments.
SDS, and 1.1% Triton X-100; it was then divided into 200-μl aliquots. One aliquot (10%) was retained as an input sample for normalization of the DNA quantity in each sample. Each aliquot was immunoprecipitated overnight at 4°C using antibodies, as mentioned in the figure legends. Preimmune IgG was used as a negative control. The antibody–protein complex was collected by adding protein A–agarose/salmon sperm DNA (50% slurry; Sigma Chemicals) for 1 h at 4°C. The protein A–agarose/antibody/protein complex was washed to remove DNA fragments that were bound nonspecifically. DNA was eluted from the beads, cross-linking was reversed, and after purification the DNA was analyzed by real-time PCR amplification of the mouse Akt1 promoter (−600 to +1). Data were normalized with the corresponding input values.

RESULTS

Increased Expression of Akt1 in Cells Subjected to Mitochondrial Stress

The relative levels of mtDNA and nuclear DNA in control, mtDNA-depleted, and reverted cells are shown in Figure S1. mtDNA content in cells treated with EtBr was reduced by ~80% relative to that in control cells. We also used 2,3′-ddC to deplete mtDNA by ~70% compared with control cells (Figure S1). In reverted cells, mtDNA levels were within 20% of control levels. Unless specified, EtBr-induced mtDNA-depleted cells, hereafter referred to as mtDNA-depleted cells, have been used in experiments reported here. The 2,3′-ddC–depleted cells are referred to as ddC mtDNA-depleted cells.

Akt is overexpressed in tumorigenic cells (Plas and Thompson, 2005); therefore we assessed Akt levels after mtDNA depletion. Figure 1A (top) shows the Akt protein level in total lysates from control, mtDNA-depleted cells, and CCCP-treated control cells. Cells subjected to mitochondrial stress via mtDNA depletion or CCCP treatment showed twofold to threefold higher Akt protein relative to controls. In reverted cells, the protein level was close to that in control cells (Figure 1A, bottom), indicating the increase in Akt is a direct response to mitochondrial respiratory stress.

Akt1 mRNA in C2C12 cells was increased twofold in mtDNA-depleted and CCCP-treated cells, compared with control cells (Figure 1B). In reverted cells, Akt1 mRNA was reduced to a level closer to the control cells (1.5-fold above control). The differences in theAkt2 mRNA levels between the cell types were not significant, indicating that Akt2 in these cells is not regulated by mitochondrial stress.

The generality of Akt1 induction in response to mitochondrial stress was further investigated using nontransformed cells (mouse embryonic fibroblasts [MEF] and RAW264.7 macrophages) and transformed cells (A549 lung carcinoma, H9C2 cardiac myoblasts, and MCF-7 breast cancer). As shown in Figure 1C, the level of Akt1 mRNA was increased in MEF cells in response to stress inducer, CCCP. In A549 cells (Figure 1D), H9C2 cells (Figure 1E) and RAW264.7 cells, both the steady-state level of Akt in the total lysate and the level of phosphorylated Akt (Ser 473) were increased in response to mtDNA depletion or CCCP treatment. In the case of MCF-7 cells (Figure 1G), only the level of phosphorylated Akt but not the steady-state Akt increased in response to CCCP treatment. These results show that an increase in steady-state level of Akt under mitochondrial stress is cell specific, although an increase in Ser-473 phosphorylation/activation of Akt1 is a common response to mitochondrial stress in many cell types.

Mitochondrial Stress Results in Activation and Increased Nuclear Localization of Akt

Akt1 exists as a soluble protein in the cytosol and translocates to the nucleus under growth-stimulatory conditions (Trotman et al., 2006). As shown in Figure 2A, steady-state Akt1 levels in the cytosol and nucleus were increased threefold in mtDNA-depleted cells, compared with control cells. 2,3′-ddC–induced mtDNA depletion also caused nuclear accumulation of Akt1 (Figure S1D). Akt activity was increased 2.5-fold in total lysate and nuclear fractions of mtDNA-depleted cells (Figure 2B), but was increased only marginally in the cytosolic fraction.

Akt activation is dependent on phosphorylation by PDK1 (Ser473) and PDK2 (Thr308) (Chen et al., 2001; Kondapaka et
Tyrosine phosphorylation is also important for Akt activation. Nuclear extracts from control and mtDNA-depleted cells were immunoprecipitated using Akt antibody (Figure 2C, top), and the immunoprecipitates were probed with phospho-specific antibodies. Ser473-specific antibody yielded a more intense band with nuclear extract from mtDNA-depleted cells (three- to fourfold higher), compared with control cell extract (Figure 2C). This difference is consistent with the nearly threefold increase of Akt activity in nuclear extract from mtDNA-depleted C2C12 cells (see Figure 2B). Thr308-directed antibody yielded ~50% higher intensity in extract from mtDNA-depleted cells, relative to that from control cells. The specificity of antibody-based detection of phospho-Akt was confirmed using agents known to inhibit phosphorylation of Akt at Ser473 and Thr308. ML9 markedly reduced staining with anti-Ser473 antibody, and UCNO1 reduced the band intensity with Thr308 antibody (Figure S2). Steady-state protein levels were not affected by these inhibitors.

**Akt1 Induction under Mitochondrial Stress Is Dependent on Cn/IGF1 Receptor/Phosphoinositide-3 Kinase Pathway**

Mitochondrial stress signaling is initiated by Cn activation, which in turn activates IGF1 receptor downstream. We showed previously that inhibiting IGF1R activity makes mtDNA-depleted cells susceptible to apoptosis. We studied the role of IGF1R in the activation of Akt1, which is known to promote cell survival. We used siRNA-targeted silencing of CnAα and IGF1R to investigate the role of Cn/IGF1R in Akt1 induction under mitochondrial stress (Figure 3, A–C). Transient transfection with siRNA for CnAα and IGF1R reduced the respective mRNA levels by 60–80%, relative to levels in mock-transfected (scrambled siRNA) cells (Figure S3, A and B). Akt1 mRNA levels were reduced 40% by CnAα mRNA silencing in mtDNA-depleted cells, but were not changed in control and reverted cells (Figure 3A). IGF1R mRNA silencing caused an 80% reduction in Akt1 mRNA in mtDNA-depleted cells and 10% reduction in reverted cells, but no change in control cells (Figure 3B). Akt1 protein levels were also reduced significantly in mtDNA-depleted cells after either CnAα or IGF1R mRNA silencing (Figure 3C), but neither reduced Akt1 protein in control cells (data not shown).

As shown for other Tyr kinase receptors, IGF1R-dependent Akt activation may involve phosphoinositide-3 kinase (PI3-K) activity. This possibility was tested by treating control and mtDNA-depleted cells with the PI3-K-specific inhibitor LY294002 and measuring the levels of p-Akt (phospho-Ser473) in the nuclear fraction. To compare p-Akt levels, we used equal amounts of input Akt protein from the control and mtDNA-depleted cells. LY294002 treatment resulted in a significant decrease (50%) in p-Akt levels in control and mtDNA-depleted cells, suggesting Akt activation is a PI3-K-dependent process (Figure 3D).

**Transcriptional Activation of Akt1 Promoter in Response to Mitochondrial Stress**

We next examined if the increase in Akt1 mRNA in mtDNA-depleted cells was due to transcriptional activation of the Akt1 promoter, using a human Akt1 promoter DNA-reporter construct (Park et al., 2005). Nucleotide sequence analysis (MatInspector) indicated that the 900-base pairs region of the Akt1 gene upstream of the transcription start site contains consensus sites for binding to the stress-activated transcription factors NFκB c-Rel, C/EBPβ, and CREB (Figure 4A), similar to what has been shown for other stress target genes (Biswas et al., 2005b; Guha et al., 2009). The promoter activity in mtDNA-depleted cells was nearly fourfold higher than in control cells (Figure 4A). Although not shown, 5′ deletion analysis revealed that this minimal promoter region was sufficient to respond to mitochondrial stress–mediated transcription activation. Cotransfection of the promoter-reporter construct with c-Rel, C/EBPβ, and hnRNP A2 cDNAs resulted in 2-, 3-, and 1.5-fold higher promoter driven luciferase activity, respectively, over the basal activity without cDNA transfection. As shown recently for other stress-response genes (Guha et al., 2009), cDNA transfections in control C2C12 cells increased promoter activity only marginally. Cotransfection with hnRNP A2 cDNA alone induced the promoter activity by ~60%. Cotransfections with hnRNP A2 cDNA and either c-Rel or C/EBPβ cDNAs resulted in a three- to fourfold increase in luciferase activity, suggesting a cooperative interaction between these factors (Figure 4A). Akt1 promoter activity was markedly lowered in hnRNP A2-silenced mtDNA-depleted cells (Figure 4B), confirming the role of hnRNP A2 in Akt1 transcription. This is consistent with the reduced nuclear Akt1 protein levels found in hnRNP A2-silenced mtDNA-depleted cells (Figure 5A). In keeping with our observations with other stress responsive target genes (RYR1, CnL, and Glut-4), these results suggest that hnRNP A2 may function as a coactivator of Akt1 transcriptional activity under mitochondrial stress.

The induction of stress signaling activation of Akt1 is not restricted to EtBr-treated cells because ddC mtDNA-depleted C2C12 cells also showed markedly increased nuclear

**Figure 3.** The role of calcineurin, IGF1R and PI3-K in the activation of Akt1 in cells subjected to mitochondrial stress. (A) Effects of CnAα mRNA knockdown on the levels of Akt1 mRNA in control, mtDNA-depleted, and reverted C2C12 cells. (B) Effects of IGF1R mRNA knockdown on the levels of Akt1 mRNA in control, mtDNA-depleted, and reverted C2C12 cells. In both A and B, Akt1 mRNA levels were quantified using real-time PCR analysis. Mean ± SEM values were calculated from three separate estimates. (C) Immunoblot analysis of total cell lysates (50 μg each) from control, mtDNA-depleted C2C12 cells, and control as well as depleted cells expressing siRNAs to CnAα and IGF1R were developed with Akt antibody. A companion blot was probed with actin antibody to assess loading levels. (D) Effects of the PI3-K inhibitor LY294002 (50 μM, 2 h) on Akt and phospho-Ser473 Akt in control and mtDNA-depleted cells. The immunoblot represents the analysis of total lysates (50 μg protein each) developed with the indicated antibodies. A companion blot was probed with actin antibody to assess protein loading.
Akt and also phosphorylated Akt (Figure S7A). Additionally, important marker genes of stress signaling such as RYR1, CnL, IGF1R, hnRNPA2, and Akt are also transcriptionally up-regulated in ddC mtDNA-depleted cells as seen by increase in mRNA levels by 2–3.5-fold (Figure S7B).

Increased Akt1 Promoter Occupancy of Stress-activated Transcription Factors in mtDNA-depleted Cells

The in vivo association of stress activated transcription factors and hnRNPA2 with the Akt1 promoter DNA was investigated by ChIP analysis. The promoter occupancy of these factors in response to mitochondrial respiratory stress was compared in control C2C12 cells, mtDNA-depleted cells, and mtDNA-depleted cells in which hnRNPA2 mRNA was silenced (Figure 4C). As shown in Figure 4C, the footprint of the mouse promoter for stress-specific factor-binding motifs is similar to the human Akt1 promoter, in that the proximal 600-base pair region contains sites for binding to c-Rel, CREB, and C/EBPδ as in the human Akt1 promoter, although the relative order of these factors differs for the two promoters. The mouse promoter also contains a site for CREB immediately upstream of the transcription start site. ChIP analysis in Figure 4 shows that the occupancies of the Akt1 promoter by hnRNPA2, C/EBPδ, c-Rel, and p50 were 10-, 30-, 7- and 6-fold higher, respectively, in mtDNA-depleted C2C12 cells compared with control cells. hnRNPA2 silencing in mtDNA-depleted cells markedly reduced the levels of these factors, confirming that hnRNPA2 is an adaptor protein that facilitates the recruitment of NFκB, c-Rel/p50, and C/EBPδ to the transcription complex.

Interaction of Nuclear Akt1 and hnRNPA2 in mtDNA-depleted Cells

We investigated the physical interaction of Akt1 with hnRNPA2 using an antibody pulldown method, and protein species in the immunoprecipitates were identified by nano-liquid chromatography (LC)/mass spectrometry (MS) analysis. Figure S5A shows the gel pattern of nuclear proteins from control, mtDNA-depleted, and mtDNA-depleted/hnRNPA2 knockdown cells immunoprecipitated by Akt1 antibody. Those protein bands that were more abundantly pulled down from mtDNA-depleted cell extracts compared with control and hnRNPA2 knockdown cell extracts (Figure S5A, arrows) were subjected to LC-MS analysis. Most of the putative Akt1-interacting proteins identified are functionally related to cell morphology, cell survival, resistance to apoptosis, and regulation of oxidative stress (Table 1). Interestingly, the identification of hnRNPA2 as a putative Akt1-interacting protein was most relevant to this article. Notably, low or negligible levels of hnRNPA2 were pulled down from control and mtDNA-depleted/hnRNPA2 knockdown cells, whereas a major population of hnRNPA2 was identified in mtDNA-depleted cells. This was confirmed by immunoblot analysis of protein pulled down by Akt1 antibody. Akt1 antibody pulled down hnRNPA2 in high abundance from mtDNA-depleted cells, but only negligible amounts were precipitated from the control nuclear

Table 1. Nuclear proteins associated with Akt in mtDNA-depleted cells

| Protein          | Molecular weight (kDa) |
|------------------|------------------------|
| Thioredoxin      | 12                     |
| Transgelin       | 22                     |
| Myosin           | 120                    |
| Actin            | 43                     |
| 14-3-3ε          | 29                     |
| Annexin A2       | 37                     |
| HnRNPA2          | 36                     |
| HnRNPK           | 65                     |
| Vimentin         | 57                     |
| Galectin 3       | 31                     |
| Tubulin          | 50                     |

Distinctive protein bands immunoprecipitated by Akt antibody, as shown in Supplementary Figure S5, were excised and subjected to nano LC-MS/MS analysis.
extract (Figure 5A). Other proteins pulled down from mtDNA-depleted cells (Table 1; e.g., thioredoxin, transgelin, and galectin 3 are involved in tumorigenesis, and some are known substrates of Akt1).

We also observed significantly higher levels of Ser-, Thr-, and Tyr-phosphorylation of nuclear hnRNPA2 in mtDNA-depleted cells (Figure 5B). Sequence analysis (using www.phosphoscan.mit.edu) of hnRNPA2 (Figure 5C, top) revealed two consensus sites for Akt phosphorylation, at Thr98 and Ser219, and four potential Tyr phosphorylation sites at Y123, Y264, Y275, and Y288. We further investigated the role of Akt1 phosphorylation of hnRNPA2 in this study. However, the role of Tyr phosphorylation was not investigated further.

The autoradiogram in Figure 5C of an in vitro kinase assay using recombinant hnRNPA2 shows that hnRNPA2 is phosphorylated in vitro by Akt1 kinase. We investigated the significance of the Ser219 and Thr98 sites for Akt1-dependent phosphorylation using bacterially expressed and purified wild-type (WT) and mutant hnRNPA2 proteins (T98A and S219A). In vitro translation (Figure S6A), as well as the gel pattern of the purified recombinant proteins (Figure S6B), showed no difference in either translation efficiency or purity between WT, T98A, and S219A mutant proteins. Figure 5D shows the gel profiles of purified hnRNPA2 incubated with and without Akt1 in presence of 10 μCi [γ-32P]ATP. Results show that phosphorylation is diminished by ∼90% in the T98A mutant and by almost 70% in the S219A mutant, relative to WT protein. These results suggest that both Thr98 and Ser219 are targets for Akt-mediated phosphorylation and demonstrate that Akt1 phosphorylates hnRNPA2 both under in vitro and in vivo conditions.

The functional significance of Akt1 phosphorylation of hnRNPA2 was further investigated by mutational analysis. Previously we have shown that WT hnRNPA2 with c-Rel or C/EBPβ cooperatively stimulated transcription activation of CnL and RyR1 promoters in mtDNA-depleted cells. Cells were cotransfected with or without WT and mutant (T98A and S219A) hnRNPA2 cDNAs and c-Rel, C/EBPβ cDNAs as indicated. CLP, cathepsin L promoter. Transfection conditions were as described in Figure 4 and Materials and Methods.
We investigated the role of Akt1 on the transcription-activator function of hnRNPA2, using ChIP analysis of CnL promoter region in C2C12 cells expressing scrambled siRNA (mtDNA-depleted/mock siRNA) and mtDNA-depleted C2C12 cells expressing Akt1 siRNA (mtDNA-depleted/Akt1siRNA). Figure 6A shows levels of Akt1 mRNA levels in mtDNA-depleted/mock siRNA and mtDNA-depleted/Akt1siRNA cells. Goat IgG was used as a negative antibody control. (C) ChIP analysis of CnL promoter using hnRNPA2 antibodies in control cells, compared with Akt1 knockout MEFs treated with and without CCCP (25 μM, 5 h). Goat IgG was used as an antibody control. Values have been normalized to 10% of the corresponding input values. (D) Promoter activity of CnL promoter-reporter construct (1.0 μg) in MEFs co-transfected with different cDNAs (0.2 μg) as indicated. Values represent the mean ± SEM of three separate assays. Renilla luciferase activity was used for normalization of transfection efficiency as described in Materials and Methods.

The role of Akt1 as an activator of hnRNPA2 was investigated further by cotransferring the CnL promoter construct with various cDNAs in control and Akt1-knockout MEFs, with or without CCCP treatment for 5 h. Treatment of control MEFs with CCCP increased the transcriptional activity of the CnL promoter by 2.7-fold, compared with the untreated control cells (Figure 6D). Promoter activities in Akt1 knockout cells were markedly lower than in control cells, and in the former case, CCCP treatment had no effect on promoter activity. In control MEF cells treated with CCCP, cotransfection with c-Rel, C/EBPβ, and hnRNPA2 increased promoter activity by 4.4-, 4.0-, and 1.4-fold, respectively, over that in untreated control cells. Cotransfections in Akt1 knockout cells had minimal to marginal effects even after CCCP treatment. Cotransfection with a combination of c-Rel plus hnRNPA2, or C/EBPβ plus hnRNPA2, increased activity more than eightfold in control cells treated with CCCP, compared with untreated control cells. Similar cotransfections in Akt1-knockout cells had no effect on promoter activity, with or without added CCCP. These results support the role of Akt1 in the transcriptional activation of stress-response genes and the promoter occupancy of hnRNPA2.
Akt1 Silencing Results in the Reversal of Invasive Phenotype and Induces Apoptosis in mtDNA-depleted Cells

We showed previously that increased invasive behavior and resistance to apoptosis are hallmarks of cells subjected to mitochondrial respiratory stress in different cell types (Amuthan et al., 2001, 2002; Biswas et al., 2005a). Because Akt1 activation is associated with cell tumorigenicity (Semenza et al., 2001; Deberardinis et al., 2008), we tested the effects of Akt1 silencing on apoptosis and in vitro invasiveness in mtDNA-depleted cells. Figure 7A shows the percent apoptosis in control, mtDNA-depleted, and reverted C2C12 cells after Akt1 silencing. All three cell types contain ~2-5% apoptotic cells when Akt1 expression is unaltered (mock siRNA). Akt1 mRNA silencing increased the apoptotic cell population to ~70% in mtDNA-depleted cells, but a much smaller effect was seen in control and reverted cells (4–8%).

Figure 7B shows the in vitro Matrigel invasion pattern of control, mtDNA-depleted, and reverted C2C12 cells. As reported previously, mtDNA-depleted cells showed a remarkably higher level of invasiveness compared with control and reverted C2C12 cells. Interestingly, Akt1 mRNA silencing reduced the invasiveness of mtDNA-depleted cells by ~85%. However, Akt1 mRNA silencing had a marginal effect on control and reverted cells. Because equal numbers of viable cells were used in all three cases, these results show Akt1 plays an important role in the invasive behavior of C2C12 cells subjected to mitochondrial respiratory stress, possibly by regulating the activity of hnRNPA2 and, therefore, the transcriptional activity of stress response genes.

DISCUSSION

The Ser/Thr kinase Akt/PKB is modulated by multiple intra- and extracellular stimuli, including hypoxia, heat shock, oxidative stress, and cytokines (Datta et al., 1997; Semenza et al., 2001; Lawlor and Alessi, 2001; Cho et al., 2001; Deberardinis et al., 2008). Akt is involved in a multitude of cellular processes, including cell survival, cell proliferation, cancer progression, cell metabolism, RNA transport, and transcriptional modulation (Summers and Birnbaum, 1997; Brunet et al., 1999; Datta et al., 1999; Whiteman et al., 2002; Hu et al., 2005; Huang and Chen, 2005; Lee et al., 2008). Akt activation is often coupled to a change in metabolism and a shift to increased glucose uptake and glycolysis. Recent studies have shown that cells subjected to mitochondrial stress develop resistance to apoptosis, which is accompanied by a shift in cellular metabolism (Amuthan et al., 2001; Lee et al., 2004; Meierhofer et al., 2004; Guha et al., 2007; Kulawiec et al., 2009). The metabolic shift to glycolysis involves a switch from insulin- to IGF1 receptor-mediated signaling in C2C12 cells (Guha et al., 2007). We have also shown that IGF1 receptor phosphorylation may be an autocrine process because these cells secrete increased IGF1 (Guha et al., 2007). Preliminary results also suggest that the activation involves IRS1 phosphorylation. In this study, we show the metabolic shift is accompanied by increased nuclear accumulation and activation of Akt under mitochondrial stress.

Previous studies showed activation of Akt in mtDNA-depleted tumor cells (Pelicano et al., 2006; Moro et al., 2009), although the precise mechanisms of activation were not...
elucidated. Using respiration-deficient human leukemia and colon cancer cells, Pelicano et al. (2006) suggested that Akt activation in response to mitochondrial stress may be due to increased mitochondrial NADH accumulation and inactivation of PTEN through a redox regulated mechanism. The nature of this redox regulation remains unclear. More recently, Moro et al. (2009) showed that in mtDNA-depleted prostate epithelial cells, Akt activation was associated with up-regulation p85 and Pi3-K. Using mRNA knockdown in mtDNA-depleted C2C12 cells and CCCP treatment of control and Akt1 knockout MEFs, we show that Akt1 activation is critical for the propagation of mitochondrial respiratory stress signaling. Akt1 is activated by multiple pathways. We show here that Akt1 activation in mtDNA-depleted or CCCP-treated C2C12 cells is dependent on activation of Cn, IGF1R, and Pi3-K (see Figure 8A), critical hallmarks of stress signaling. Based on our previous results on mechanism of IGF1R activation, AMPK pathway is not likely involved in this activation (Guha et al., 2007). Although not shown, mitochondrial stress induced activation of Akt in A549, H9C2, MEF, and MCF-7 cells also was dependent on IGF1R and Pi3-K activation. Here, we extend previous observations, both in terms of the mechanism of stress-induced Akt1 activation and its role in transcriptional activation of nuclear target genes and also identify hnRNPA2 as a novel target for Akt action.

It is well recognized that Akt mediates transcription activation directly via phosphorylation and activation of transcription coactivators, such as c300 (Huang and Chen, 2005) and Forkhead transcription factors such as Foxo3a (Brunet et al., 1999). We show here that Akt1 phosphorylates hnRNPA2 in vitro at Thr98 and/or Ser219, and that Akt1 activity in vivo is essential for the recruitment of the transcription coactivator hnRNPA2 to the enhanceosome complex. This possibility is further supported by immunoprecipitation experiments showing that antibody to Akt1 pulled down hnRNPA2 and a number of other nuclear proteins from mtDNA-depleted cells. Negligible or undetectable precipitation of this protein by Akt1 antibody from control and reverted cell extracts suggest the Akt1-hnRNPA2 interaction is predominately a respiratory stress-related process. Some of the other proteins pulled down by Akt1 antibody from mtDNA-depleted cells, such as actin, tubulin, and 14-3-3, are known substrates for Akt1 (Vandermoere et al., 2007). At present the functional significance of the association of Akt1 with other proteins in Table 1 and their relevance to mitochondrial stress remains unclear.

Multiple techniques, including differential mRNA display, cDNA array, and proteomics, have revealed that a wide spectrum of genes encoding mitochondrial respiratory complex proteins, cell surface receptors, and proteins involved in apoptosis, Ca2+ homeostasis, and transcription regulation are either up- or down-regulated in cells lacking or partially depleted of mtDNA (Delseit et al., 2002; Crimi et al., 2005; Jahangir Tafrechi et al., 2005; Biswas et al., 2008; Johnston et al., 2009). Our results on the stress-induced expression of Akt1 mRNA in cells subjected to mitochondrial stress by mtDNA depletion, as well as by CCCP treatment, were surprising in view of studies showing the regulation of human Akt1 gene expression by the Src/Stat3 pathway by Park et al. (2005), who have shown convincingly that Stat3 response elements are localized in the exon 1-intron 1 region of the human Akt1 gene (Park et al., 2005). It is becoming apparent that many genes undergo multimodal regulation under the influences of different transcription activator proteins and coactivators. For example, Glut-4 expression is known to involve cMyc and other factors (Thai et al., 1998), whereas mitochondrial respiratory stress-induced transcriptional up-regulation involves c-Rel/p50, C/EBPβ, CREB, and hnRNPA2 (Guha et al., 2009). Interestingly, respiratory stress-induced transcriptional activation of the human Akt1 promoter involves the same set of transcription factors, and the overall location of putative DNA motifs for these factors within the 900-base pairs region immediately upstream of transcription initiation site resembles those of other stress target genes (Guha et al., 2009). Notably, the footprint of stress response elements for the human Akt1 promoter is conserved in the mouse Akt1 gene.

Our data advances understanding of the process by which mitochondrial respiratory stress signaling modulates the transcription of nuclear genes. This study not only describes the mechanism of stress-induced activation of Akt1, but also defines its role in the phosphorylation and activation of the transcriptional coactivator hnRNPA2. Importantly, the activation of Akt1 in response to mitochondrial respiratory stress involves the same four signature factors (C/EBPβ, c-Rel/p50, NFAT, and CREB) and recruitment of hnRNPA2. Previously we have shown that different domains of hnRNPA2 may be involved in the binding or recruitment of different transcription factors (Guha et al., 2009). In view of this mutational analysis, in this study showing the importance of the Akt target sites of hnRNPA2 (T98 and S219) suggests that these two phosphorylation sites may be important for recruiting different transcription factors. The observed 60–70% reduction in activity with either T98A or S219A mutant hnRNPA2 is consistent with our proposed model on the cooperativity between the signature factors (c-Rel/p50, C/EBPβ, CREB and NFAT) in the activation of stress target genes (Guha et al., 2009 and Figure 8).

An intriguing observation is the self-regulatory role of Akt1, in that transcriptional activation of this gene requires Akt1-mediated phosphorylation of hnRNPA2. The general mechanism of stress-induced transcription activation is presented as a model in Figure 8B. We believe the mechanism described here is important in understanding the cellular response to altered mitochondrial function and bioenergetics.

ACKNOWLEDGMENTS

We thank Drs. G. Dreyfuss (University of Pennsylvania, Philadelphia, PA) for the human Akt1-producing cell line, N. G. Avadhani (University of Florida, Gainesville, FL) for the human Akt1 promoter, Shankar Ghosh (Columbia University Medical Center, NY) for c-rel cDNA, and Michael Atchison (University of Pennsylvania) for C/EBPβ cDNA. We also thank Satish Srinivasan (University of Pennsylvania) for the mtDNA-depleted RAW264.7 cells and Li Huang for technical help. This research was supported by National Institutes of Health Grants RO1 CA-22762 to N.G.A. and RO1DK-56886 to M.J.B.

REFERENCES

Amuthan, G., Biswas, G., Ananadatheerthavarada, H. K., Vijayasarathy, C., Shephard, H. M., and Avadhani, N. G. (2002). Mitochondrial stress-induced calcium signaling, phenotypic changes and invasive behavior in human lung carcinoma A549 cells. Oncogene 21, 7839–7849.

Amuthan, G., Biswas, G., Zhang, S. Y., Klein-Szanto, A., Vijayasarathy, C., and Avadhani, N. G. (2001). Mitochondria-to-nucleus stress signaling induces phenotypic changes, tumor progression and cell invasion. EMBO J. 20, 1910–1920.

Arnould, T., Vankoningsloo, S., Renard, P., Houblon, A., Ninane, N., Demazy, C., Remacle, J., and Raes, M. (2002). CREB activation induced by mitochondrial dysfunction is a new signaling pathway that impairs cell proliferation. EMBO J. 21, 53–63.
Babcock, D. F., Herrington, J., Goodwin, P. C., Park, Y. B., and Hille, B. (1997). Mitochondrial participation in the intracellular Ca\(^{2+}\) network. J. Cell Biol. 136, 833–844.

Biswas, G., Adebajo, O. A., Freedman, B. D., Anandatheerthavarada, H. K., Vijayarasarathy, C., Zaidi, M., Kotlikoff, M., and Avadhani, N. G. (1999). Retrograde Ca\(^{2+}\) signaling in C2C12 skeletal myocytes in response to mitochondrial genetic and metabolic stress: a novel mode of inter-organelle crosstalk. EMBO J. 18, 522–533.

Biswas, G., Anandatheerthavarada, H. K., and Avadhani, N. G. (2005a). Mechanism of mitochondrial stress-induced resistance to apoptosis in mitochondrial DNA-depleted C2C12 myocytes. Cell Death Differ. 12, 266–278.

Biswas, G., Anandatheerthavarada, H. K., Zaidi, M., and Avadhani, N. G. (2003). Mitochondria to nucleus stress signaling: a distinctive mechanism of NF\(\kappa\)B Rel activation through Cr-mediated inactivation of IkappaBalpha. J. Biol. Chem. 278, 507–519.

Biswas, G., Guha, M., and Avadhani, N. G. (2005b). Mitochondria-to-nucleus stress signaling in mammalian cells: nature of nuclear gene targets, transcription regulation, and induced resistance to apoptosis. Gene 354, 132–139.

Biswas, G., Tang, W., Sondheimer, N., Guha, M., Bansal, S., and Avadhani, N. G. (2008). A distinctive physiological role for IkappaB in the propagation of mitochondrial respiratory stress signaling. J. Biol. Chem. 283, 12586–12594.

Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Bennis, J., and Greenberg, M. E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96, 857–868.

Chen, R., Kim, O., Yang, J., Sato, K., Eisenmann, K. M., McCarthy, J., Chen, H., and Yu, H. (2001). Regulation of Akt/PKB activation by tyrosine phosphorylation. J. Biol. Chem. 276, 31858–31862.

Cho, H., Mu, J., Qian, C., Crenshaw, E. B., III, Kaestner, K. H., Bartolomei, M. S., Shulman, G. I., and Birnbaum, M. J. (2001). Akt mediates tumor suppressor p53-dependent apoptosis in cultured prostate carcinoma cells. Cancer Cell. 19, 3173–3183.

Dey, R., and Moraes, C. T. (2000). Lack of oxidative phosphorylation and low mitochondrial membrane potential strongly inhibit the protective effect of Bcl-x(L) in osteosarcoma cells. J. Biol. Chem. 275, 20302–20308.

Dey, R., and Moraes, C. T. (2000). Lack of oxidative phosphorylation and low mitochondrial membrane potential strongly inhibit the protective effect of Bcl-x(L) in osteosarcoma cells. J. Biol. Chem. 275, 20302–20308.

Delsite, R., Kachhap, S., Anbazhagan, R., Gabrielson, E., and Singh, K. K. (1997). Akt phosphorylation of BAD couples survival signals to the mitochondrial stress-induced resistance to apoptosis. J. Biol. Chem. 272, 11491–11501.

Dey, R., and Moraes, C. T. (2000). Lack of oxidative phosphorylation and low mitochondrial membrane potential strongly inhibit the protective effect of Bcl-x(L) in osteosarcoma cells. J. Biol. Chem. 275, 20302–20308.

Dey, R., and Moraes, C. T. (2000). Lack of oxidative phosphorylation and low mitochondrial membrane potential strongly inhibit the protective effect of Bcl-x(L) in osteosarcoma cells. J. Biol. Chem. 275, 20302–20308.

Dey, R., and Moraes, C. T. (2000). Lack of oxidative phosphorylation and low mitochondrial membrane potential strongly inhibit the protective effect of Bcl-x(L) in osteosarcoma cells. J. Biol. Chem. 275, 20302–20308.

Dey, R., and Moraes, C. T. (2000). Lack of oxidative phosphorylation and low mitochondrial membrane potential strongly inhibit the protective effect of Bcl-x(L) in osteosarcoma cells. J. Biol. Chem. 275, 20302–20308.

Dey, R., and Moraes, C. T. (2000). Lack of oxidative phosphorylation and low mitochondrial membrane potential strongly inhibit the protective effect of Bcl-x(L) in osteosarcoma cells. J. Biol. Chem. 275, 20302–20308.

Dey, R., and Moraes, C. T. (2000). Lack of oxidative phosphorylation and low mitochondrial membrane potential strongly inhibit the protective effect of Bcl-x(L) in osteosarcoma cells. J. Biol. Chem. 275, 20302–20308.

Dey, R., and Moraes, C. T. (2000). Lack of oxidative phosphorylation and low mitochondrial membrane potential strongly inhibit the protective effect of Bcl-x(L) in osteosarcoma cells. J. Biol. Chem. 275, 20302–20308.

Dey, R., and Moraes, C. T. (2000). Lack of oxidative phosphorylation and low mitochondrial membrane potential strongly inhibit the protective effect of Bcl-x(L) in osteosarcoma cells. J. Biol. Chem. 275, 20302–20308.

Dey, R., and Moraes, C. T. (2000). Lack of oxidative phosphorylation and low mitochondrial membrane potential strongly inhibit the protective effect of Bcl-x(L) in osteosarcoma cells. J. Biol. Chem. 275, 20302–20308.

Dey, R., and Moraes, C. T. (2000). Lack of oxidative phosphorylation and low mitochondrial membrane potential strongly inhibit the protective effect of Bcl-x(L) in osteosarcoma cells. J. Biol. Chem. 275, 20302–20308.

Dey, R., and Moraes, C. T. (2000). Lack of oxidative phosphorylation and low mitochondrial membrane potential strongly inhibit the protective effect of Bcl-x(L) in osteosarcoma cells. J. Biol. Chem. 275, 20302–20308.

Dey, R., and Moraes, C. T. (2000). Lack of oxidative phosphorylation and low mitochondrial membrane potential strongly inhibit the protective effect of Bcl-x(L) in osteosarcoma cells. J. Biol. Chem. 275, 20302–20308.
Patry, C., Bouchard, L., Labrecque, P., Gendron, D., Lemieux, B., Toutant, J., Lapointe, E., Wellinger, R., and Chabot, B. (2003). Small interfering RNA-mediated reduction in heterogeneous nuclear ribonucleoparticule A1/A2 proteins induces apoptosis in human cancer cells but not in normal mortal cell lines. Cancer Res. 63, 7679–7688.

Pelicano, H., et al. (2006). Mitochondrial respiration defects in cancer cells cause activation of Akt survival pathway through a redox-mediated mechnism. J. Cell Biol. 175, 913–923.

Petros, J. A., et al. (2005). mtDNA mutations increase tumorigenicity in prostate cancer. Proc. Natl. Acad. Sci. USA 102, 719–724.

Plas, D. R. and Thompson, C. B. (2005). Akt-dependent transformation: there is more to growth than just surviving. Oncogene 24, 7435–7442.

Semenza, G. L., et al. (2001). ‘The metabolism of tumours’: 70 years later. Novartis Found. Symp. 240, 251–260.

Shidara, Y., Yamagata, K., Kanamori, T., Nakano, K., Kwong, J. Q., Manfredi, G., Oda, H., and Ohta, S. (2005a). Positive contribution of pathogenic mutations in the mitochondrial genome to the promotion of cancer by prevention from apoptosis. Cancer Res. 65, 1655–1663.

Shidara, Y., Yamagata, K., Kanamori, T., Nakano, K., Kwong, J. Q., Manfredi, G., Oda, H., and Ohta, S. (2005b). Positive contribution of pathogenic mutations in the mitochondrial genome to the promotion of cancer by prevention from apoptosis. Cancer Res. 65, 1655–1663.

Singh K. K., Ayasamy, V., Owens, K. M., Koul, M. S., and Vujic, M. (2009). Mutations in mitochondrial DNA polymerase-gamma promote breast tumorigenesis. J. Hum. Genet. 54(9):516–524.

Summers, S. A. and Birnbaum, M. J. (1997). A role for the serine/threonine kinase, Akt, in insulin-stimulated glucose uptake. Biochem. Soc. Trans. 25, 981–986.

Taylor, R. W. and Turnbull, D. M. (2005). Mitochondrial DNA mutations in human disease. Nat. Rev. Genet. 6, 389–402.

Thai, M. V., Guruswamy, S., Cao, K. T., Pessin, J. E., and Olson, A. L. (1998). Myocyte enhancer factor 2 (MEF2)-binding site is required for GLUT4 gene expression in transgenic mice. Regulation of MEF2 DNA binding activity in insulin-deficient diabetes. J. Biol. Chem. 273, 14285–14292.

Trotman, L. C., Alimonti, A., Scaglioni, P. P., Koutcher, J. A., Cordon-Cardo, C., and Pandolfi, P. P. (2006). Identification of a tumour suppressor network opposing nuclear Akt function. Nature 441, 523–527.

Vandermoere, F., El Yazidi-Belkoura, I., Demont, Y., Slomianny, C., Antol, J., Lemoine, J., and Hondermarck, H. (2007). Proteomics exploration reveals that actin is a signaling target of the kinase Akt. Mol. Cell Proteom. 6, 114–124.

Whiteman, E. L., Cho, H., and Birnbaum, M. J. (2002). Role of Akt/protein kinase B in metabolism. Trends Endocrinol. Metab. 13, 444–451.

Yamaoka, K., Imajoh-Ohmi, S., Fukuda, H., Akita, Y., Kurosawa, K., Yamamoto, Y., and Sanai, Y. (2006). Identification of phosphoproteins associated with maintenance of transformed state in temperature-sensitive Rous sarcoma-virus infected cells by proteomic analysis. Biochem. Biophys. Res. Commun. 343, 1240–1246.

Yu, M., Shi, Y., Wei, X., Yang, Y., Zang, F., and Niu, R. (2009). Mitochondrial DNA depletion promotes impaired oxidative status and adaptive resistance to apoptosis in T47D breast cancer cells. Eur. J. Cancer Prev. 18, 445–457.