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Biochimica et Biophysica Acta 1763 (2006) 1108–1124

BIOCHIMICA ET BIOPHYSICA ACTA

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Structural and functional consequences of c-N-Ras constitutively associated with intact mitochondria

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Received 25 May 2006; received in revised form 19 July 2006; accepted 27 July 2006
Available online 12 August 2006

Abstract

We demonstrate that both c-N-Ras and c-K(B)-Ras are constitutively associated with purified mitochondria. c-K(B)-Ras is associated with the mitochondrial outer membrane, and c-N-Ras is associated with both the outer membrane and inner mitochondrial compartments. The mitochondrial morphology is abnormal in both c-N-Ras negative and K-Ras negative cells. Normal mitochondrial morphology was restored by targeting N-Ras to both the inner and outer mitochondrial compartments, or by ectopically expressing c-K(B)-Ras. Impaired mitochondrial function can result in increased CHOP and NFκB activity, typical for a retrograde signaling response. Both are constitutively elevated in the N-Ras negative cells, but not in the K-Ras negative background, and are restored by c-N-Ras targeted exclusively to the inner mitochondrial compartment. Surprisingly, both targeting and the ability to functionally reduce retrograde transcriptional activity were found to be independent of c-N-Ras farnesylation. Overall, these data demonstrate for the first time a (1) farnesylation independent function for c-N-Ras and (2) that N-Ras within the inner mitochondrial compartment is an essential component of the retrograde signaling system between the mitochondria and nucleus.

Keywords: Ras; Retrograde signaling; Mitochondrial stress; Morphology; Transcriptional activity

1. Introduction

The immediate family of Ras GTPases consists of three genes and four proteins, Ha-, N-, K(A)-and K(B)-Ras, with the latter two generated through alternative splicing of the K-Ras gene. Ras proteins cycle between inactive GDP bound and active GTP bound forms. Ras proteins interact with target proteins that bind to the three-dimensional effector domains (Switch I and II regions) of Ras through their RBDs (Ras binding domains). The four Ras protein isoforms, Harvey, N, K (A) and K(B), contain identical sequences in the Switch I and Switch II domains that comprise the Ras effector domain and can thus bind all identified effector or target proteins albeit with differing affinities. The C-terminal hypervariable regions of the Ras proteins contain no homology other than the last four amino acids comprising the CAAX box which is prenylated on the cysteine residue followed by proteolytic cleavage of the 3 remaining C-terminal residues. Additional post-translational modifications following the initial farnesylation and proteolysis include sequential carboxy-methylation and palmitoylation. One exception is that K(B)-Ras can be geranylgeranylated and is not palmitoylated since the poly-lysine stretch serves to anchor the protein through interaction with polar head groups of membrane phospholipids. It is important to note that the highest degree of posttranslational similarity is between c-N-Ras and c-K(A)-Ras, each getting farnesylated and possessing only a single site of palmitoylation. Oncogenic Ras proteins possess mutations that result in a higher proportion of the molecules being in the active versus the inactive form. The function(s) of Ras proteins have, until recently, been described in the context of acute signaling transduction, stimulation of quiescent cells with a growth factor or other agent that promotes rapid changes in intracellular cascades, usually culminating in the changes in gene expression [1–4]. Previously published work from this laboratory demonstrated that both c-N-Ras and c-K(B)-Ras

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doi:10.1016/j.bbamcr.2006.07.015

possess steady-state functions in addition to their well-characterized roles in acute signal transduction. c-N-Ras provides a steady-state anti-apoptotic signal while c-K(B)-Ras is solely responsible for the steady-state production of MMP-2 [5,6].

The paradigm that Ras proteins exist attached exclusively to the plasma membrane has given way to more recent data demonstrating the presence of Ras proteins localized to subcellular compartments including the mitochondrion [7], Golgi apparatus [8–11] and the endoplasmic reticulum (ER) [8,10,11]. The functional significance and consequence of Ras signaling on endomembranes versus the plasma membrane is still unclear. Other groups have reported transient movement of Ras isoforms to the mitochondrial structure [7,12]. There is also data suggesting that several Ras binding partners reside within intact mitochondria [13–16], suggesting the possibility of Ras-dependent signaling originating from within mitochondrial membranes. Ras localized to endomembranes can respond to growth factor challenges similar to plasma membrane associated Ras [8,10,17,18], though their endogenous functions and the relationships of their localization to specific endomembranes structures have not been clearly defined. These data suggest that each Ras isoform might regulate multiple signaling pathways and biological outcomes that are defined by their specific subcellular locations.

Coordinate expression of nuclear and mitochondrial-encoded subunits was first observed during the biogenesis and replication of mitochondria [19–21]. A second level of mitochondria-to-nuclear communication likely arose out of necessity. Mitochondrial function declines with age, in both yeast and mammalian organisms. This decline in mitochondrial function necessitates a compensatory mechanism for ATP synthesis, usually the upregulation of glycolytic enzymes [22]. This adaptive communication from mitochondria to the nuclear genome has been termed 'retrograde signaling (or response)'. *S. cerevisiae* was found to have three positive regulators of retrograde signaling (Rtg1, 2, and 3) and four negative regulators (Mks1p, 14-3-3, Bmh1p and Bmh2p). Rtg 1 and 3 form a transcription factor complex that translocates to the nucleus and affects gene expression. The translocation of the Rtg1:3 complex depends on the level of association of Rtg 2 with the negative regulators [23–27]. The integrity of the yeast retrograde response was also shown to be dependent on the function of the RAS2 gene [28]. In mammals, retrograde signaling has been examined using two different models. The first being removal of the mitochondrial DNA, generating ρ^0 cells that are defective in electron transport and a more mild situation, one in which the mitochondria accumulate a denatured form of ornithine transcarbamylase (an unfolded protein response) [22,29–33]. Both 'mitochondrial stress' situations resulted in significant changes in the activity of specific nuclear transcription factors, CREB, NFκB and CHOP (Gadd153), respectively. Cells containing dysfunctional mitochondria can adapt, by increasing nuclear-expression of genes encoding for metabolic proteins, small molecule transport proteins and by increasing the biogenesis of peroxisomes [34]. These adaptations allow yeast to survive in the absence of ETC function. One consequence of this adaptation, however, is that those organisms are generally smaller and metabolically slower. On the other hand, in the absence of ETC function, the significant decrease in ROS content results in extending their life-span [22,28,35]. These observations suggest that there is an innate ability of the mitochondria to communicate its 'status' to the nucleus, which then makes the necessary adjustment for either (1) just survival or (2) optimal growth.

Mitochondrial regulation of such transcription factors as CREB, CHOP and NFκB suggest that the extra-mitochondrial signaling is mediated through traditional signal transduction intermediates. In fact, mitochondrial activation of CREB occurs through a PKA-dependent phosphorylation event, analogous to its activation through plasma membrane receptor-mediated events [30]. Other groups have reported transient movement of Ras isoforms to the mitochondrial structure [7]. There is also data demonstrating that several Ras binding partners also reside within intact mitochondria [14–16], suggesting the possibility of a Ras-dependent signaling system originating from within mitochondrial membranes.

In this report we present data suggesting that both c-N-Ras and c-K(B)-Ras each contribute to normal mitochondrial morphology. We also demonstrate that c-N-Ras is localized to both the outer mitochondrial membrane and inner mitochondrial compartment. The localization of c-N-Ras to the inner compartment is independent of its well-defined posttranslational modifications. In addition, c-N-Ras within the inner mitochondrial compartment appears to be an integral component of the mitochondrial retrograde signaling system, regulating both CHOP and NFκB activity.

2. Materials and methods

2.1. Cell culture

N-Ras knockout (N−/−), heterozygote (N+/−), and control N+/+ mouse embryo fibroblasts (MEFs) were a generous gift from R. Kucherlapati (Albert Einstein College of Medicine) [36]. K-Ras knockout and control K+/+ MEFs were a generous gift from T. Jacks (Howard Hughes Medical Institute, Massachusetts Institute of Technology) [37]. MEFs were immortalized by a modification of the 3T3 protocol [38]. The MEFs were passaged 1:3 every 7 days until they developed a fibroblast morphology. To avoid any cell-specific changes arising from immortalization, multiple, independently isolated cell lines were used throughout these studies. Cells were grown in high glucose DMEM containing 10% FBS (Atlanta Biologicals, Lawrenceville, GA), non-essential amino acids (GIBCO/Invitrogen, Carlsbad, CA) and antibiotic/antimycotic (GIBCO/Invitrogen, Carlsbad, CA). Cells were grown in a humidified incubator containing 10% CO_2. Transfections were performed with Lipofectamine, Plus Reagent and Opti-MEM I (Invitrogen, Carlsbad, CA) as directed in product literature. Selections were performed with 400 μg/ml G418 Sulfate (Mediatech, Herndon, VA) and stable clones were maintained in 200 μg/ml G418.

2.2. Antibodies

Antibodies against Raf-1, Histone H1, N-Ras, K(A)-Ras, and K(B)-Ras were from Santa Cruz Biotechnology (Santa Cruz, CA). Porin and IP3R1 antibodies were from Calbiochem/EMD Biosciences (Darmstadt, Germany). Na,K-ATPase and GM-130 antibodies were from BD Transduction Laboratories (San Diego, CA). LAMP-2 antibody was from AbCam (Cambridge, MA). Myc antibody was from Upstate Cell Signaling Solutions (Charlottesville, VA). F_1-ATPase antibody was a generous gift from M. Yamaguchi (Scripps Institute).

2.3. Whole cell extract

Cells were rinsed and scraped into PBS and collected by centrifugation (500×*g* for 5 min) at 4 °C. Cells were resuspended in p21 buffer (20 mM MOPS, 5 mM $MgCl_2$, 0.1 mM EDTA, 200 mM sucrose, pH 7.4) containing 1% CHAPS, phosphatase and protease inhibitors (30 mM β-glycerophosphate, 5 mM p-nitrophenyl phosphate, 1 mM each phosphoserine and phosphothreonine, 0.2 mM phosphotyrosine, 100 μM sodium orthovanadate, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 25 μg/ml pepstatin A, and 1 mM PMSF). The cell lysates were centrifuged at 13,000×*g* for 10 min to remove nuclei and cell debris. The supernatants were collected for further analysis. Protein concentration was determined by the method of Bradford.

2.4. Sucrose density gradient analysis

Cells (~3×10^7) were rinsed and scraped into PBS and collected by centrifugation (500×*g* for 5 min) at 4 °C. Cells were washed two times in p21 buffer. Cells were resuspended in a minimum p21 buffer (~300 μl) containing phosphatase and protease inhibitors and allowed to swell on ice for 30 min. Cells were broken using a Dounce homogenizer until greater than 90% were trypan blue positive. The whole cell homegenate was carefully layered onto a 5–60% continuous sucrose gradient (in 20 mM MOPS, 5 mM $MgCl_2$, 0.1 mM EDTA, pH 7.4 in the presence of the previously described protease and phosphatase inhibitors). The samples were centrifuged in an SW41 rotor for 2 h at 45,000×*g*. The gradient was fractionated into ~0.5 ml samples with 100 μl of each fraction analyzed for the indicated proteins by SDS-PAGE separation and immunoblot analysis.

2.5. Mitochondria purification

Cells were rinsed and scraped into PBS and collected by centrifugation (500×*g* for 5 min) at 4 °C. Cells were washed two times in p21 buffer. Cells were resuspended in p21 buffer containing phosphatase and protease inhibitors and allowed to swell on ice for 30 min. Cells were lysed using a Dounce homogenizer until ~90% were trypan blue positive. The homogenate was centrifuged at 900×*g* for 5 min at 4 °C to pellet nuclei and unbroken whole cells. The supernatant was centrifuged at 7700×*g* for 10 min at 4 °C to pellet heavy membranes. The supernatant was removed and the pellet was resuspended in a maximum of 1 ml p21 buffer containing 10 mM EDTA. This was applied to the top of a discontinuous gradient (from bottom to top): 2.3 ml 2.5 M sucrose in 15 mM MOPS (pH 7.4), 6.6 ml 100% Percoll, and 12.25 ml p21 buffer, without sucrose, with 10 mM EDTA. The gradients were centrifuged at 62,000×*g* for 45 min at 4 °C. The pancake of mitochondria, at the 2.5 M sucrose/Percoll interface, was transferred to a new tube and was diluted with equal amounts of p21 buffer (with 10 mM EDTA) and p21 buffer (no sucrose, with 10 mM EDTA). Diluted mitochondria were centrifuged at 9000×*g* for 15 min at 4 °C [39]. The purified mitochondria were washed in p21 buffer. Mitochondria were resuspended in p21 buffer containing 1% CHAPS, phosphatase and protease inhibitors. The mitochondrial lysates were centrifuged at 13,000×*g* for 10 min. The supernatants were collected and used for further analysis. Protein concentration was determined by the method of Bradford [40].

2.6. Inner and outer mitochondrial membrane separation

Purified pelleted mitochondria (not CHAPS solubilized) were resuspended in 1 ml 1 mM MOPS pH 7.4. The volume of pellet was measured and 50× pellet volume of lysis buffer (1 mM MOPS, 0.25 mM EDTA, 0.25 mM EGTA, pH 7.4) was added. Mitochondria were stirred on ice for 3 min. Mitochondria were gently homogenized with 2–3 strokes to separate the inner membrane (IM) from the outer membrane (OM). The homogenized mitochondria were returned to stirring on ice for 17 min. An ultracentrifuge tube was filled 1/3 full with 35% sucrose in 10 mM Tris pH 7.4. The mitochondrial homogenates were diluted in lysis buffer to the volume required to fill the remaining volume in the ultracentrifuge tube. (Samples were centrifuged at 100,000×*g* for 75 min at 4 °C in a swinging bucket rotor (SW41). The red/brown pellet was the inner mitochondrial membrane/matrix. The layer at the sucrose interface was the outer mitochondrial membrane. Each was removed, diluted with p21 buffer without sucrose and collected by centrifugation at 100,000×*g* in a mini-ultracentrifuge. Pellets were solubilized in p21 buffer plus 1% CHAPS.

2.7. Plasma membrane/P100 fraction

Cells were rinsed and scraped into PBS and collected by centrifugation (500×*g* for 5 min) at 4 °C. Cells were washed two times in p21 buffer. Cells were resuspended in p21 buffer containing phosphatase and protease inhibitors and allowed to swell on ice for 30 min. Cells were broken using a Dounce homogenizer until approximately 90% were trypan blue positive. The homogenate was centrifuged at 900×*g* for 5 min at 4 °C to pellet nuclei and whole cells. The supernatant was centrifuged at 13,000×*g* for 30 min at 4 °C to pellet heavy membranes. The supernatant was retained and centrifuged at 100,000×*g* for 1 h. The enriched plasma membrane pellet was resuspended in p21 buffer containing 1% CHAPS, phosphatase and protease inhibitors. The supernatants were collected for further analysis. Protein concentration was determined by the method of Bradford [40].

2.8. Mitochondrial targeting

Mitochondrial Inner Membrane: c-N-Ras was targeted to the inner mitochondrial membrane with the pShooter mammalian expression vector system (Invitrogen, Carlsbad CA). c-N-Ras was subcloned into the pEF-myc-mito vector which targets proteins to the inner mitochondrial membrane using the COX VIII cDNA N-terminal targeting sequence. Mitochondrial Outer Membrane: c-N-Ras was targeted to the outer mitochondrial membrane using the mitochondrial targeting sequence of OMP25 [41]. The OMP25 transmembrane construct was a kind gift from Pietro De Camilli, Yale University. The transmembrane domain of OMP25 (amino acids 178–200) was inserted, in frame, on the C-terminus of c-N-Ras such that a fusion protein was expressed, N-Ras-OMP.

2.9. Immunoblotting

Lysates containing equal amounts of protein (100 μg unless otherwise specified) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (PVDF) (Hybond P, Amersham/Pharmacia Biotech.). The membrane was blocked with Blocker Casein (Pierce Chemical Co.) in PBS containing 2% newborn calf serum (Life Technologies, Inc.-Invitrogen). The washed blots were incubated with primary antibodies (1:1000) for 2 to 3 h at room temperature or overnight at 4 °C. Following washing in TBS-0.1% Tween 20, the blots were incubated with horseradish peroxidase-labeled secondary antibodies (1:1000) for 1 h at room temperature. After washing, the signals were detected using standard enhanced chemiluminescence (ECL) techniques.

2.10. Electron microscopy

Cells were grown in regular growth media until just sub-confluent. All solutions were used at 37 °C. Media was aspirated and cells were washed with PBS. Trypsin was added to cells. Once detached, cells were washed with PBS. Cells were gently pelleted and resuspended in EM fixative (2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4). Fixed cells were washed with cold sodium cacodylate buffer and then incubated in 1% osmium tetroxide for 60 min. Cells were washed with sodium cacodylate buffer, then Maleate buffer (pH 5.1), and stained for 60 min with 1% uranyl acetate in maleate buffer. Stained cells were washed with maleate buffer and dehydrated through washes in increasing concentrations of ethanol. Cells were transferred from 100% ethanol to propylene oxide followed by 1:1 propylene oxide:Epon, then to pure Epon. 85 nm sections were cut from Epon blocks. Sections were viewed on a Phillips CM12 Electron Microscopy Scope.

2.11. Reverse transcription-PCR analysis

Total RNA was isolated from cells with Trizol (Invitrogen, Carlsbad, CA) as directed by the manufacturer. RNA pellets were resuspended in RNAse-free water and RNA concentrations were determined spectrophotometrically. 1 μg RNA was used for the reverse transcription reaction with the ImProm-II Reverse

Transcription System (poly-dT primer) from Promega (Madison, WI). Reactions were set up according to manufacturer's recommendations. Reverse transcription was performed at 25 °C for 5 min, 42 °C for 60 min, and 70 °C for 15 min. PCR amplification was performed with the Advantage-HF 2 PCR kit from BD Biosciences/Clontech (Palo Alto, CA). The denaturing temperature was 90 °C, and the annealing and elongation temperatures were 61 °C. The PCR amplification was run for 25 cycles to allow for semi-quantitative analysis. The oligonucleotide primers were synthesized by SIGMA (St. Louis, MO). The primer sequences were as described [42]: CAC ATC CCA AAG CCC TCG (CHOP (gadd153) sense primer), CTC AGT CCC CTC CTC AGC (CHOP (gadd153) antisense primer), ATC GTG GGC CGC TCT TAA GGC ACC (β-Actin sense primer), and CTC TTT GAT GTC ACG CAC GAT TTC (β-Actin antisense primer).

2.12. Luciferase reporter assay

Cells were seeded into 6-well dishes (Costar, Corning NY) at low density. Reporter constructs (c-Jun and CHOP) were from the Stratagene (La Jolla, CA) PathDetect *in vivo* Signal Transduction Pathway *trans*-Reporting System. Luciferase reporters e-selectin-luc (NF-κB reporter), and MMP-2-luc were generous gifts from (X. Li, Cleveland Clinic Foundation and D. Lovett, UCSF, respectively). The construct phRL-TK (Renilla luciferase) was from Promega (Madison, WI). ΔOTC in pCAGGS was a generous gift from N. Hoogenraad, LaTrobe University, Melbourne Australia. Constructs were transiently introduced into fibroblasts using LipofectAmine/Plus Reagent and OptiMem according to product recommendations. The amount of total DNA was kept constant using the appropriate empty vectors. The luciferase activity of the reporters were analyzed with the Dual-Luciferase Reporter Assay system (Promega, Madison, WI) and normalized to the activity of the Renilla luciferase.

3. Results

3.1. c-N-Ras and c-K(B)-Ras are localized to the mitochondria

We have previously shown that all of the c-N-Ras localized to the plasma membrane resides in a steady-state complex with Raf-1 and PKCε [43]. We have also demonstrated that c-N-Ras provides a steady-state anti-apoptotic signal through the JNK signaling pathway, not the Raf-ERK-MEK pathway [44]. Recent reports have indicated that Ras isoforms are localized in and are able to signal from endomembranes. Others have shown that Ras isoforms translocate to mitochondria under specific conditions [7,45]. Based on our previous data demonstrating a steady-state anti-apoptotic function of c-N-Ras [6,44], we designed experiments to test whether c-N-Ras was constitutively localized to intact mitochondrion. Our first set of experiments separated membrane bound organelles using a continuous sucrose density gradient centrifugation (Fig. 1). Fractions were collected and immunoblotted for the Na,K-ATPase (plasma membrane), GM130 (Golgi), Porin (mitochondria) and Histone (nuclear) to determine the relative mobility of each organelle within the sucrose gradient. Samples were also immunoblotted for c-N-Ras, c-K(A)-Ras and Raf-1. Unfortunately, the K(B)-Ras specific polyclonal antibody was unable to detect its antigen in these diluted samples. The data shown in Fig. 1A suggest that c-N-Ras is distributed throughout the cellular membrane containing organelles, with at least 3 distinct subcellular localizations. In contrast to the wide-spread distribution of c-N-Ras, c-K(A)-Ras appears to be predominantly localized to the plasma membrane fraction. To validate this observation, we designed experiments to rule out any possible non-specific redistribution of c-N-Ras during sample preparations. We prepared heavy membrane (HM) (mitochondrial enriched) and light membrane (LM) fractions from both control (N+/+) and N-Ras negative cell (N−/−) populations.

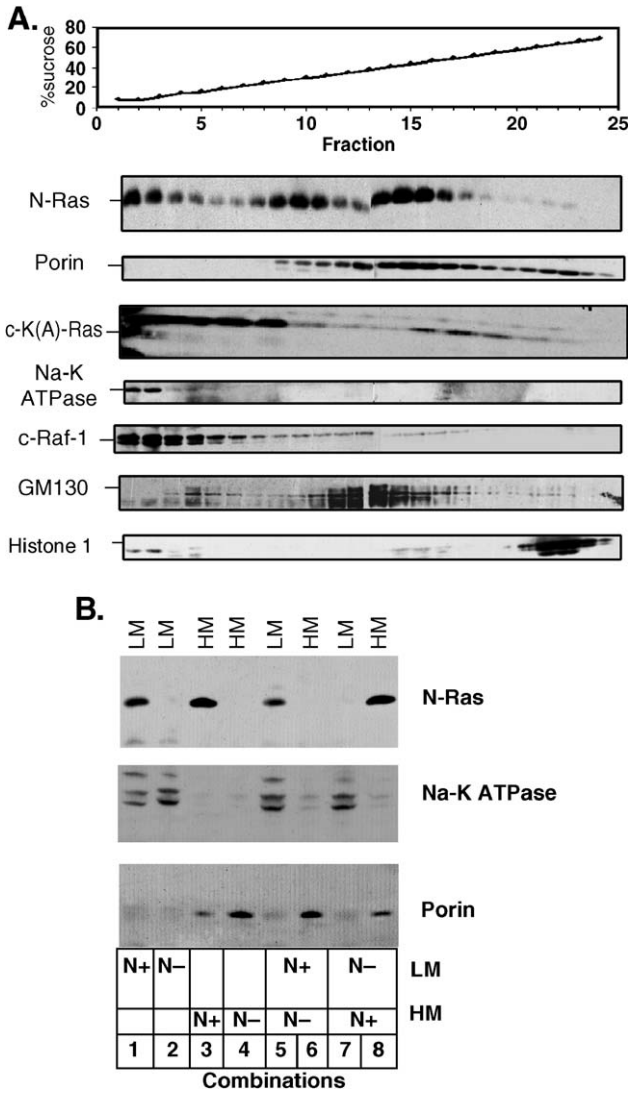


Fig. 1. N-Ras localizes to several cellular compartments. (A) 72 h serum-starved C3H10T1/2 cells were homogenized and layered onto a 5–60% continuous sucrose gradient. Following centrifugation at 45,000×*g* for 2 h, the gradient was fractionated into 0.5 ml samples. Each fraction was separated by SDS-PAGE and immunoblotted for the presence of the Na,K-ATPase, Porin, GM130 (Golgi marker), Histone, c-N-Ras, c-K(A)-Ras and Raf-1. (B) Either 10^8 control (N+/+) or N-Ras negative cells (N−/−) were scraped and lysed by Dounce homogenization. Heavy membranes of each were separated from the plasma membranes by centrifugation at 12,000×*g*. The pellets were retained as the heavy membrane fraction (HM). The remaining supernatant was used as the source of plasma or light membranes (LM). The purity of these separations was monitored by the presence of Porin for mitochondria and the Na,K-ATPase for the plasma membrane. Heavy membranes and LM fractions from N+/+ and N−/− cells were combined and left on ice for 30 min. Following this incubation, each fraction, HM and LM were re-isolated as described above. Each fraction was then immunoblotted for the presence of c-N-Ras, Porin and the Na,K-ATPase. Lanes 1–4 represent the HM and LM fractions from the indicated cell lines in the absence of any mixing. In lanes 5 and 6, the LM from the N+/+ cells was mixed with the HM from the N−/− cells. In lanes 7 and 8, the LM from the N−/− cells was mixed with the HM from the N+/+ cells.

Each fraction was correspondingly enriched in Porin (HM) and the Na-K ATPase (LM) as expected. We then mixed the fractions as shown in Fig. 1B, HM-N+/+:LM-N−/− and HM-N −/−:LM-N+/+, incubated this mixture on ice for 30 min and then re-isolated the HM and LM fractions. N-Ras was detected in fractions that originated only from N+/+ cells, without any apparent non-specific redistribution to fractions arising from the N-Ras negative background. These data rule out any possibility that the c-N-Ras localized to organelles other than the plasma membranes arises from non-specific redistribution during sample preparation. Therefore, the data presented in Fig. 1A suggest that c-N-Ras resides in at least 3 distinct subcellular pools while c-K(A)-Ras, which only differs in its posttranslational modification from c-N-Ras by the single amino acid positioning of its palmitate group, is predominantly localized to the plasma membranes.

Others have previously shown that c-N-Ras could be detected using indirect immunofluorescence in both the ER and Golgi. We designed experiments to test whether c-N-Ras was also constitutively associated with intact mitochondria. We purified mitochondria from cultured cells by subcellular fractionation as described in Materials and methods. We first determined the relative separation of plasma membrane and mitochondrial compartments by immunoblotting for mitochondria specific Porin (VDAC) versus the Na,K-ATPase as a marker for plasma membrane. Our mitochondrial preparations did not contain any measurable levels of plasma membrane contamination, though c-N-Ras was easily detected (Fig. 2A). Porin was primarily localized in the mitochondrial fraction with a small amount in the plasma membrane fraction, showing some mitochondrial contamination of the plasma membrane fraction. The lack of Porin in the whole cell lysate likely reflects its relatively low abundance compared to total cellular protein. The data presented in Fig. 2A support the idea that c-N-Ras is constitutively localized to mitochondria. We further analyzed the purity of our mitochondrial preparations using GM-130 as a marker for Golgi (Fig. 2B), LAMP-2 as a marker for late endosomes (Fig. 2B), and the IP3R1 as a marker for the ER (Fig. 2C). The immunoblot analysis shown in these figures suggest that our mitochondrial preparations are substantially free from significant contamination by other organelles. The level of c-N-Ras detected in the purified mitochondrial preparations cannot be explained by the minimal level of contamination by other organelles. As an independent validation of mitochondrial purity, we determined that the specific activity of citrate synthase was consistent with published values for purified mitochondria (1800 nmol/min per mg of protein [46] versus our preparations of 2100 nmol/min per mg protein). Taken together, these data suggest that c-N-Ras is an integral part of the mitochondrial compartment. The data shown in Fig. 1A suggest that c-K(A)-Ras should not be detected in our mitochondrial preparations. Others have also reported that K (B)-Ras was detected in the outer mitochondrial membrane [45]. We tested, therefore, whether our purified mitochondria accurately reflected these observations (Fig. 2D). Purified mitochondrial samples and enriched plasma membrane preparations were immunoblotted for both c-K(A)-Ras and c-K (B)-Ras. Consistent with the sucrose density analysis, c-K(A)-

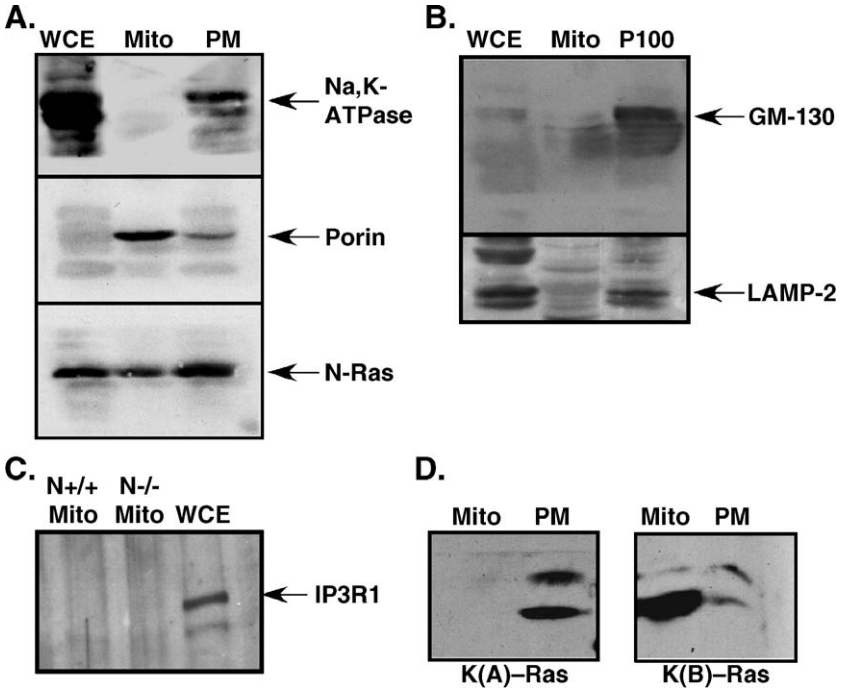


Fig. 2. c-N-Ras is detected in purified mitochondria. Whole cell extracts (WCE), purified mitochondria (Mito), and P100/plasma membrane (P100/PM) fractions were isolated as described in Materials and methods. Samples were solubilized in 1% CHAPS, equal amounts of protein were loaded onto each lane and immunoblotted for the indicated proteins. (A) Samples were analyzed for the presence of Porin (mitochondrial marker), Na,K-ATPase (plasma membrane marker) and c-N-Ras. (B) Samples were analyzed for the presence of LAMP-2 (late endosomal and lysosomal marker) and GM130 (Golgi marker). (C) Samples were analyzed for the presence of IP3R1 (endoplasmic reticulum marker). (D) Samples were analyzed for the presence of c-K(A)-and c-K(B)-Ras.

Ras was detected only in the plasma membrane fraction, though c-K(B)-Ras was found to be greatly enriched in the purified mitochondrial preparations. The failure to detect c-K(A)-Ras in purified mitochondria again demonstrate that these preparations are relatively free from plasma membrane contamination. Since the posttranslational modifications of c-N-Ras and c-K(A)-Ras are the most similar to one another of all the Ras isoforms, these data suggest that a mechanism distinct from their posttranslational modification is responsible for unique subcellular distribution of c-N-Ras.

3.2. Lack of Ras proteins alters mitochondrial morphology

The results in Fig. 2 demonstrate that both c-N-Ras and c-K(B)-Ras are constitutively associated with intact mitochondria. One of the most telling features for functional mitochondria is their morphology. Generally, mitochondria that do not display the prototypical structure with well-define cristae are functionally impaired. We utilized murine fibroblasts immortalized from control, N-Ras negative and K-Ras negative knockout embryos. We also previously demonstrated that: the wild-type cells (N+/+K+/+) express both c-N-, c-K(A)- and c-K(B)-Ras; the N-Ras knockout lines (N−/−K+/+) express both c-K(A)- and c-K(B)-Ras; and the K-Ras knockout cells (N+/+K−/−) express only c-N-Ras. None of these cell lines express detectable levels of c-Ha-Ras protein [5]. We examined the morphology of the mitochondria in wild-type and each of the Ras isoform specific knockout cell lines by electron microscopy (Fig. 3A).

The mitochondria of the wild-type cells were consistent with a normal morphology (Fig. 3A, top panels). The mitochondria were regular in shape and the appearance of the cristae was typical. This was not the case with either the N-Ras knockout (N−/−K+/+) or the K-Ras knockout (N+/+K−/−) cell lines. The mitochondria of the N-Ras knockout cells were more electron dense, generally more round in appearance and contained abnormal concentric cristae (Fig. 3A, middle panels). While some normal appearing mitochondria were present, the vast majority of the mitochondria presented this abnormal morphology. Mitochondria with similar morphology have been reported, often associated with mitochondrial diseases [47–49]. The mitochondria of the K-Ras knockout cells (N+/+K−/−) were also abnormal in appearance, though distinct from the mitochondria observed in the N-Ras negative background (Fig. 3A, bottom panels). They were generally more electron dense than the wild-type mitochondria and contained concentric cristae, however the arrangement of the concentric cristae was qualitatively different than those seen in the N-Ras knockout cells. Many of these mitochondria appeared to have small groups of concentric cristae encircled by a larger single membrane. Similar mitochondrial morphology has been noted in cells lacking expression of mitofilin [50]. As in the N-Ras knockout cells, the K-Ras knockout cells contained some normal appearing mitochondria, though the vast majority appeared abnormal in structure. The functional consequences of each Ras isoform toward respiration and generation of ROS are currently under investigation.

3.3. Restoration of mitochondrial morphology by N-Ras and K(B)-Ras

In Fig. 3A, we observed that cells lacking c-N- or c-K-Ras expression contained mitochondria with an abnormal morphology. To definitively attribute these abnormal mitochondrial structures to the absence of c-Ras isoforms, it was imperative that ectopic expression of the missing Ras isoforms restored normal mitochondrial structure. Therefore, using standard protocols, we generated stable N-Ras negative cell lines ectopically expressing c-N-Ras. We also generated stable K-Ras negative cells expressing either myc-tagged c-K(A)-Ras or c-K(B)-Ras. Expression of exogenous proteins is shown in Fig. 3B. Introduction of wild-type, c-N-Ras into the N-Ras knockout cells resulted in a near complete restoration of normal mitochondrial morphology (Fig. 3C, top panels). A few abnormal mitochondria were observed in a few cells, however the vast majority of the cells contained normal appearing mitochondria. K-Ras negative cells lack expression of both of the c-K-Ras splice products c-K(A)-Ras and c-K(B)-Ras. Ectopic expression of myc-c-K(A)-Ras did not alter the defective mitochondrial morphology in the K-Ras negative background (Fig. 3C, middle panels). Only expression of c-K(B)-Ras was able to restore a more normal mitochondrial structure (Fig. 3C, bottom panels). The cristae were restored to a normal distribution and very few cells contained any mitochondria with concentric cristae. These results are consistent with the data presented in Fig. 2, that c-K(B)-Ras and not c-K(A)-Ras is constitutively associated with intact mitochondrial structures.

3.4. Intra-mitochondrial localization of c-Ras isoforms

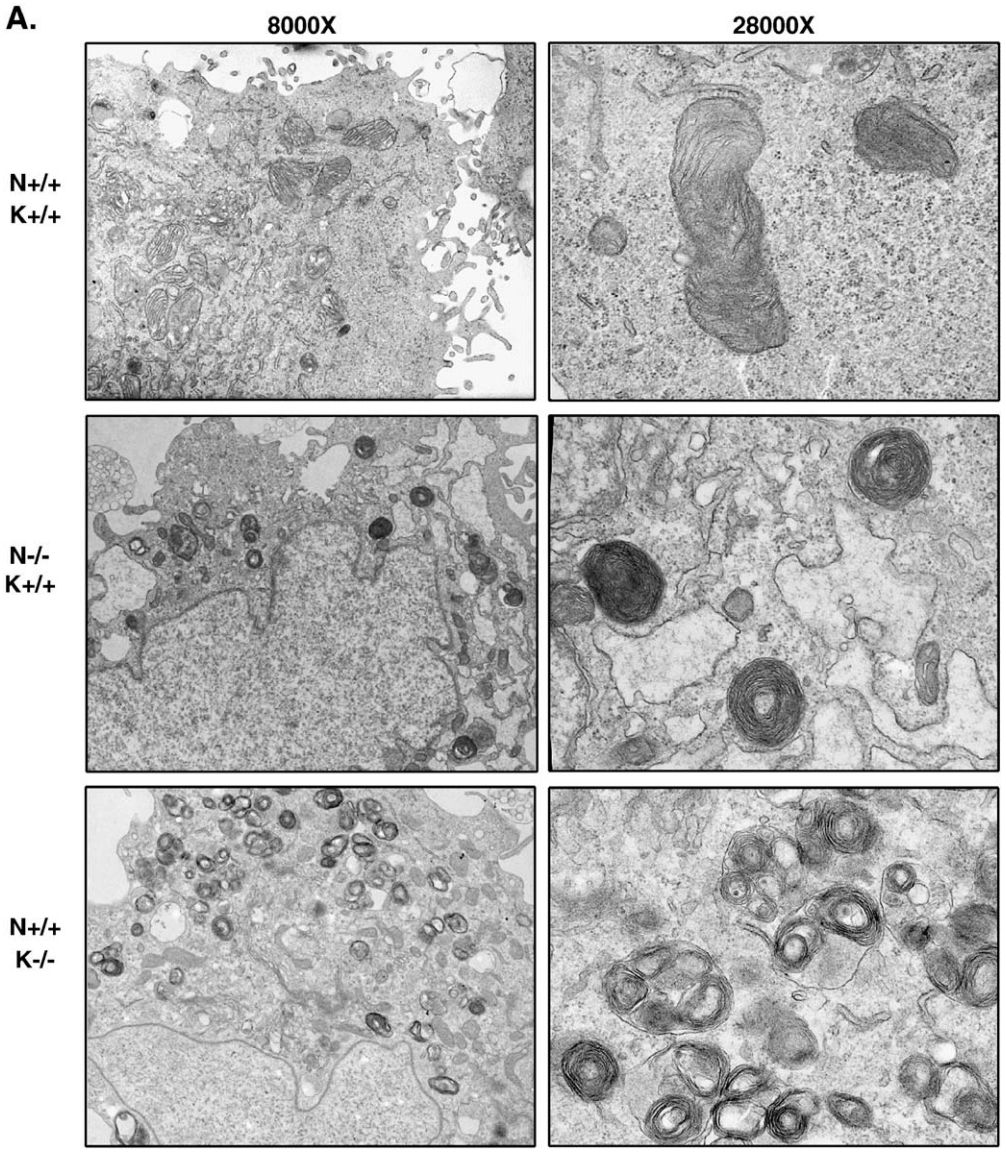
Fig. 2 established that both c-N-Ras and c-K(B)-Ras are present in intact mitochondria. We designed experiments to determine whether these proteins were present in the inner or outer mitochondrial compartments. Purified mitochondria were further separated into an outer mitochondrial membrane fraction and inner mitochondrial compartment as described in Materials and methods. CHAPS soluble extracts of each of these fractions were probed for the presence of Porin (as a marker for the outer mitochondrial membrane), c-N-Ras, and c-K(B)-Ras (Fig. 4A). Porin, as expected, was localized to the outer mitochondrial membrane. c-N-Ras was found to be present in both the inner and outer mitochondrial fractions. It should be noted that there appears to be no significant contamination of the inner mitochondrial compartment by the outer mitochondrial membranes, using the lack of detectable Porin to assess contamination. c-K(B)-Ras was determined to be present exclusively in the outer mitochondrial membrane, in agreement with work published by others [45].

We also used sensitivity to proteolytic digestion as a second, independent approach to assess the intra-mitochondrial location of both c-N-Ras and c-K(B)-Ras. Purified mitochondria were incubated in either normal buffer, buffer containing trypsin, or buffer containing both trypsin and soybean trypsin inhibitor for 30 min. Proteins in the outer mitochondrial membrane facing

outward into the cytoplasm would be cleaved by the trypsin treatment, while those in the inner compartment would be inaccessible to protease digestion. Fig. 4B shows immunoblots of these treatments probed for F_1-ATPase, an inner mitochondrial membrane protein, c-N-Ras and c-K(B)-Ras. Trypsin treatment did not affect the F_1-ATPase, as expected. c-N-Ras was partially degraded by the trypsin, indicating that at least some of the c-N-Ras is oriented in the outer mitochondrial membrane towards the cytoplasm. The c-N-Ras that was resistant to the trypsin treatment is likely to represent the proportion of the c-N-Ras that was determined to be localized to the inner mitochondrial compartment in Fig. 4A. c-K(B)-Ras was completely degraded by trypsin treatment indicative of its completely outer mitochondrial localization. As an additional control, Fig. 4C shows a similar experiment to Fig. 4B with an additional condition in which the intact mitochondria were incubated for 30 min in trypsin and 1% CHAPS. CHAPS solubilizes membranes so that the proteins which were inaccessible to the trypsin in the intact mitochondria became substrates for trypsin digestion. These data demonstrate that the F_1-ATPase and the portion of c-N-Ras that was resistant to trypsin alone was protected by intact mitochondrial membranes, and not by some other unknown, non-specific variable that prevented its degradation.

3.5. Intra-mitochondrial localization of Ras isoforms in restored cell lines

The data presented to this point demonstrate that the absence of c-N-Ras has significant consequences on mitochondrial morphology. Restoring c-N-Ras expression in the N−/− background (N−/−[c-N-Ras]) re-established normal mitochondrial morphology. It is important to validate that the ectopically expressed c-N-Ras partitions within the mitochondria identically to the endogenous protein. Certainly, one possibility is that in the absence of c-N-Ras expression, cells may have adapted to down-regulate any mitochondrial specific targeting mechanisms. Purified mitochondria from N−/−[c-N-Ras] cells were separated into inner and outer mitochondrial compartments as previously described. Porin was used as a marker for the outer



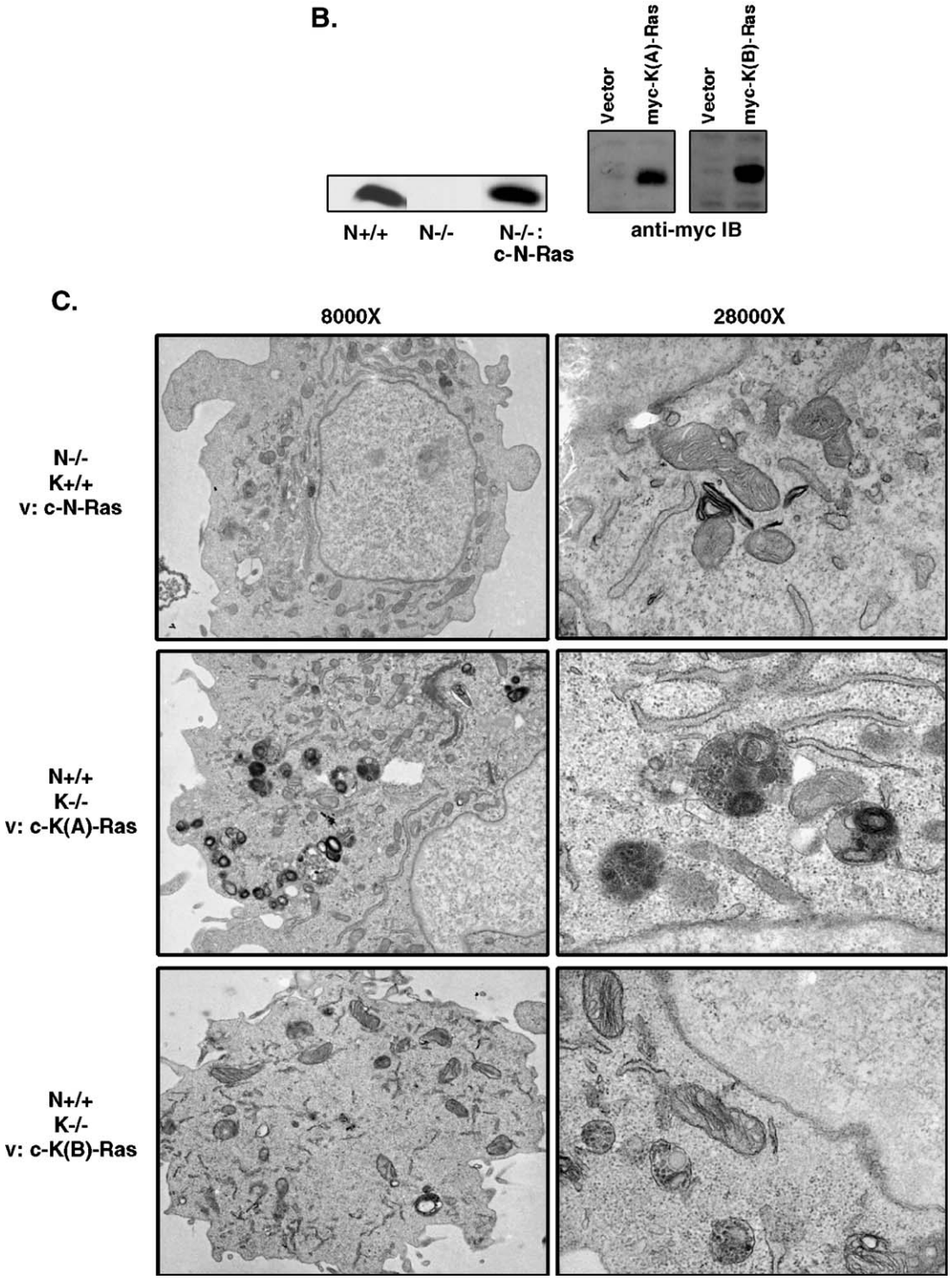


Fig. 3. N- or K-Ras expression affects mitochondrial morphology. (A) Whole cell pellets were prepared for transmission electron microscopy as described in Materials and methods. Sections of cells from wild-type (N+/+K+/+), N-Ras knockout (N−/−K+/+), and K-Ras knockout (N+/+K−/−) cells were photographed (top, middle, and bottom panels, respectively). Representative fields are shown at a magnification of 8000× (left panels) or 28,000× (right panels). (B) N-Ras negative cells were restored with expression of a c-N-Ras construct. K-Ras knockout cells were restored with expression of either an N-terminally myc-tagged c-K(A)-Ras or c-K(B)-Ras construct. Clones were isolated and expression of restored Ras protein expression was determined by immunoblot analysis. N-Ras was detected by using an anti-N-Ras specific antibody (left panel). myc-K(A)-and myc-K(B)-Ras were detected by with an anti-myc specific antibody (right panel). (C) Whole cell pellets were prepared for transmission electron microscopy as described in Materials and methods. Mitochondria from restored N-Ras knockout (N−/−K+/+c-N-Ras), K-Ras knockout cells restored with c-K(A)-Ras (N+/+K−/−c-K(A)-Ras), and K-Ras knockout cells restored with c-K(B)-Ras (N+/+K−/−c-K(B)-Ras) cells were photographed (top, middle, and bottom panels, respectively). Representative fields are shown at a magnification of 8000× (left panels) or 28,000× (right panels).

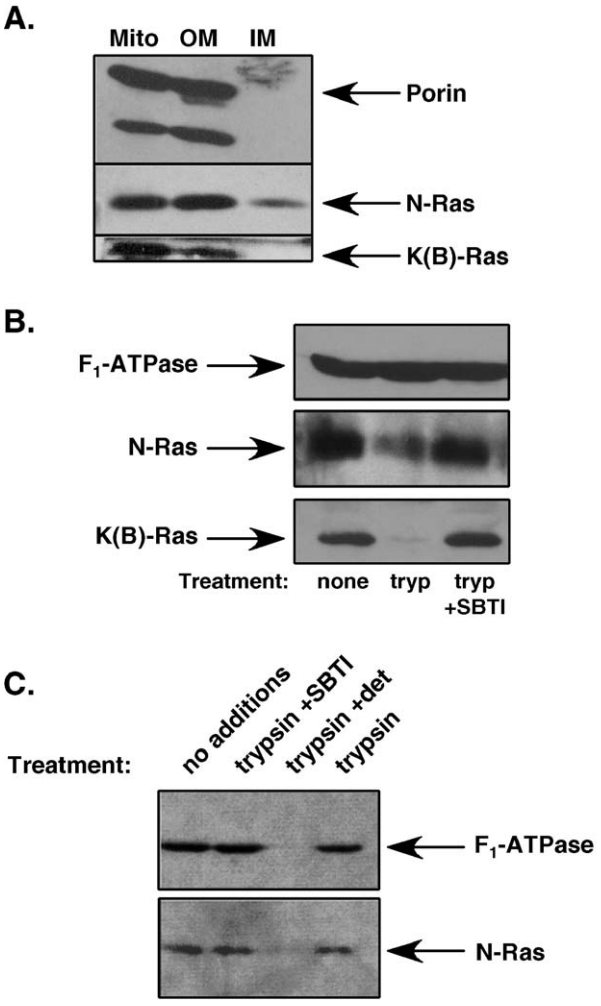


Fig. 4. Localization of endogenous N-and K-Ras to inner and outer mitochondrial membranes. (A) Mitochondria were purified from wild-type cells (Mito). The mitochondria were subjected to further purification to isolate the inner mitochondrial membranes (IM) and the outer mitochondrial membranes (OM), as described in Materials and methods. Samples were solubilized in 1% CHAPS. The presence of Porin (an outer mitochondrial membrane marker), c-N-Ras and c-K(B)-Ras localization were determined by immunoblot analysis. (B) Purified mitochondria were prepared as described. Purified mitochondria were either left untreated, treated with trypsin (tryp) or trypsin plus soybean trypsin inhibitor (tryp+SBTI) for 30 min at 30 °C. The mitochondria were recovered by centrifugation at 7700×*g*, washed and solubilized in buffer containing 1% CHAPS. Equal amounts of the detergent soluble protein were analyzed for the presence of c-N-Ras, c-K(B)-Ras, and the F_1-ATPase (inner mitochondrial membrane protein, as a negative control). (C) Purified mitochondria were treated as in B, but with an additional treatment group, trypsin in the presence of 1% CHAPS (trypsin+det). The mitochondria were recovered by centrifugation at 7700×*g*, washed solubilized in buffer containing 1% CHAPS. Equal amounts of the detergent soluble protein were analyzed for the presence of c-N-Ras and the F_1-ATPase (inner mitochondrial membrane protein, as a negative control).

mitochondrial membrane while the F_1-ATPase was used as marker for the inner mitochondrial compartment. The data shown in Fig. 5A demonstrate that ectopically expressed c-N-Ras distributes itself within the mitochondria in an identical fashion as the endogenous protein. There is no significant contamination of the inner and outer mitochondrial samples, with c-N-Ras being distributed to both compartments. The data shown in Figs. 4A and 5A might give the impression that the majority of c-N-Ras resides in the outer mitochondrial membrane. Once total protein recovery is taken into account, approximately 80% of the mitochondrial c-N-Ras resides in the inner mitochondrial compartment. In general, approximately 50–100% of the outer mitochondrial membrane sample is used for each lane in the immunoblot analysis while only 5–10% of the inner mitochondrial sample is required to load an equal amount of protein. These finds are consistent with another report that (1) GFP-N-Ras is associated with mitochondria (by immunogold detection and (2) the majority the GFP-N-Ras within mitochondrial structures is associated with the matrix [51].

Given that all previous Ras functions are reported to be dependent on its posttranslational modification, we tested whether the distribution of c-N-Ras to either the inner or outer mitochondrial compartments also required its prototypical farnesylation. N-Ras negative cells stably expressing the farnesylation defective c-N-Ras-SAAX protein were examined for N-Ras-SAAX within intact mitochondria and in the inner and outer mitochondrial compartments (Fig. 5B). As shown in Fig. 5B, the vast majority of the N-Ras SAAX was found to be cytosolic, as reported by others [52]. No cytosolic wild-type N-Ras was detected. Surprisingly, however, N-Ras-SAAX was detected in purified mitochondria and in the inner mitochondrial compartment, though not the outer mitochondrial membrane. This is the first demonstration of a farnesylation independent targeting mechanism for any of the cellular Ras isoforms. To design experiments to test the functional consequence of c-N-Ras in the inner mitochondrial compartment, we also generated an N-Ras construct that was specially targeted to the inner mitochondrial compartment. We utilized the mitochondrial targeting vector supplied by *Invitrogen* in their pSHOOTER collection. This vector uses the N-terminal targeting sequence of the subunit VIII of cytochrome C oxidase. This targeting sequence was fused to the N-terminus of both wild-type c-N-Ras (COX-N-Ras) and c-N-Ras SAAX (COX-N-Ras-SAAX). N-Ras negative cells stably expressing these constructs were analyzed for the subcellular and sub-mitochondrial localization of these N-Ras constructs (Fig. 5C). Both COX-N-Ras and COX-N-Ras-SAAX were localized exclusively to the inner mitochondrial compartment. The doublet in the COX-N-Ras samples likely arises from the intra-mitochondrial farnesylation through COX10, a farnesyl transferase essential for the proper assembly of the cytochrome C oxidase complex. The singlet observed in the COX-N-Ras-SAAX samples aligns with the top band in the COX-N-Ras samples, suggesting that separation of the farnesylated and unfarnesylated forms is easily determined by mobility within the 15% PAGE analysis. Differences in mobility between the N-Ras localized to the inner and outer mitochondrial compartments were never observed (data not shown), suggesting that the N-Ras within the inner mitochondrial compartment is processed identically to N-Ras in other cellular compartments, farnesylation followed by proteolysis and palmitoylation. Using these stable cell lines will allow us to isolate any potential function N-Ras within the inner mitochondrial compartment.

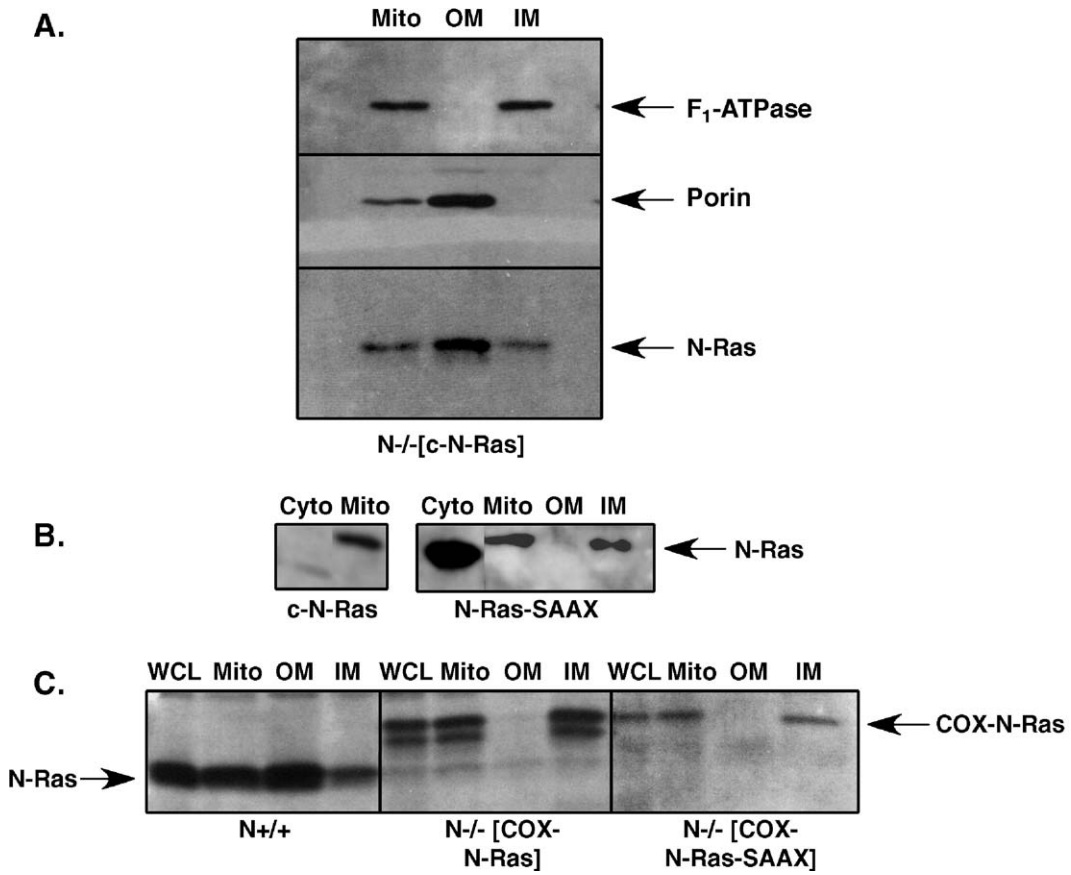


Fig. 5. Localization of exogenous Ras to inner and outer mitochondrial membranes. (A) Mitochondria were purified from N-Ras knockout cells restored with c-N-Ras (Mito). The mitochondria were subjected to further purification to isolate the inner mitochondrial membranes (IM) and the outer mitochondrial membranes (OM), as described in Materials and methods. Samples were solubilized in 1% CHAPS and analyzed for localization of F_1-ATPase (an inner mitochondrial membrane marker), Porin (an outer mitochondrial membrane marker), and c-N-Ras by immunoblot analysis. (B) Mitochondria were purified from N-Ras knockout cells restored with either c-N-Ras or N-Ras-SAAX. The mitochondria were subjected to further purification to isolate the inner mitochondrial membranes (IM) and the outer mitochondrial membranes (OM), as described in Materials and methods. Cytoplasmic fractions (supernatants from a 100,000×*g* centrifugation) were also obtained from both cell types. Samples were solubilized in 1% CHAPS and analyzed for localization of N-Ras by immunoblot analysis. (C) Whole cell lysates (WCL), whole mitochondria (Mito), outer mitochondrial membranes (OM) and inner mitochondrial membranes (IM) were purified from wild-type (N+/+), and N-Ras knockout cells restored with COX-N-Ras or COX-N-Ras-SAAX targeted to the inner mitochondrial membranes. Samples were solubilized in 1% CHAPS and analyzed for localization of N-Ras by immunoblot analysis.

3.6. Localization of N-Ras affects mitochondrial morphology

We next examined the role of c-N-Ras in the inner and outer mitochondrial compartments on mitochondrial morphology. EM micrographs of the COX-N-Ras cells are shown in Fig. 6A (top panel). Considering that the majority of the mitochondrial localized c-N-Ras is found within the inner mitochondrial compartment, we were surprised that ectopic expression of COX-N-Ras had no significant restorative effect on the morphology of mitochondria within N-Ras negative cells. We considered that this might suggest that c-N-Ras within the outer mitochondrial membrane might have a potential effect on mitochondrial morphology. To examine this, we generated an N-Ras construct specifically targeted to the outer mitochondrial membrane. Others have reported that the C-terminal targeting sequence of OMP25 (outer mitochondrial membrane protein of 25 kDa) specifically targets proteins to the mitochondrial membrane through its transmembrane domain [41,53]. N-Ras negative cells stably expressing N-Ras-OMP were generated as described for COX-N-Ras. Subcellular localization and mitochondrial distribution experiments demonstrated that N-Ras-OMP was indeed detected in the outer mitochondrial membrane (data not shown), as reported by others [41,53]. In parallel transfections, we also generated N-Ras negative cells that stably expressed both COX-N-Ras and N-Ras-OMP. The expression of N-Ras-OMP, as with COX-N-Ras, had no significant restorative effects on mitochondrial morphology (Fig. 6A: middle panels). Co-expression of both constructs, however, restored normal appearing mitochondria with easily discernable cristae. These data suggest that N-Ras localized to both the inner and outer mitochondrial compartments contribute functions that impinge on normal mitochondrial morphology.

We also noted that in addition to the obvious differences in mitochondrial structure, there appeared to be a significant difference in overall mitochondrial size. Relative mitochondrial size was measured using the 8000× magnifications as described in Materials and methods. The data are shown in Fig. 6B as a box plot that accounts for the inherent heterogeneity of

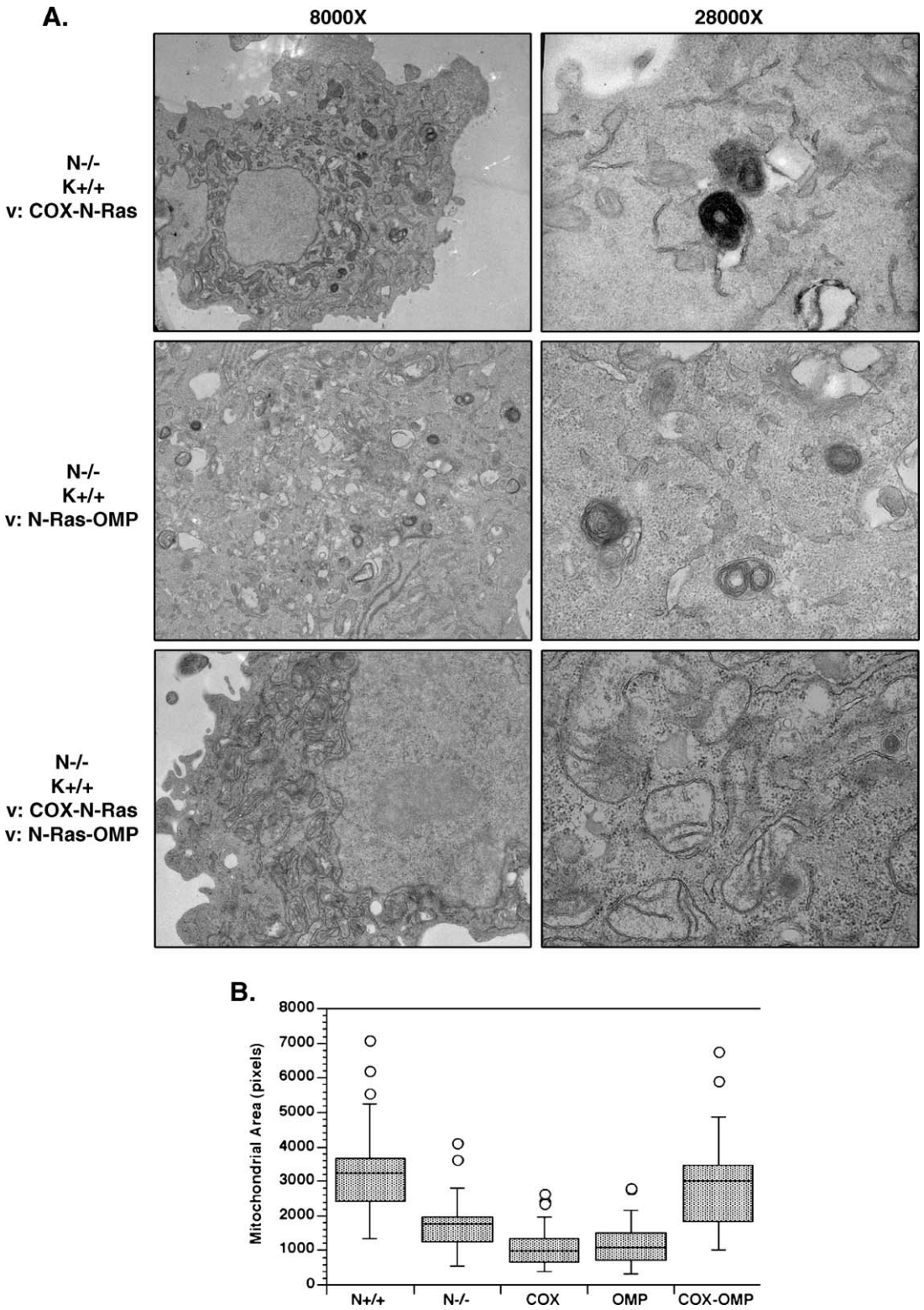


Fig. 6. Restoration of mitochondrial morphology by N-Ras. (A) Whole cell pellets were prepared for transmission electron microscopy as described in Materials and methods. Mitochondria from N-Ras knockout cells restored with inner mitochondrial membrane-targeted (N−/−K+/+COX-N-Ras), outer mitochondrial membrane targeted (N−/−K+/+N-Ras-OMP), or both inner and outer mitochondrial membrane targeted (N−/−K+/+COX-N-Ras N-Ras-OMP) N-Ras were photographed (top, middle, and bottom panels, respectively). Representative fields are shown at a magnification of 8000× (left panels) or 28,000× (right panels). v: refers to the vector encoding the indicated N-Ras construct. (B) TEM micrographs of wild-type (N+/+), N-Ras knockout (N−/−), N-Ras knockout cells restored with inner mitochondrial membrane-targeted (COX), outer mitochondrial membrane targeted (OMP), or both inner and outer mitochondrial membrane targeted (COX-OMP) N-Ras at a magnification of 8000× were scanned. Average area of individual mitochondria within each cell type was calculated using ImagePro Plus software. The range and distribution of mitochondrial area across populations is represented by a box and whisker plot. In this plot, the box represents the middle 50% of the data, the end of the upper whisker represents the 75th percentile and the end of the lower whisker represents the 25th percentile of the populations. The mean is represented by the line inside of the data box. The statistical outliers of each data set are represented by circles outside of the whisker area. Between 40 and 120 individual mitochondria, from different cells, were used for the statistical analysis.

mitochondrial size. The N−/−, COX-N-Ras and N-Ras-OMP cell lines possess mitochondria that are significantly smaller in overall size than within the N+/+ control cells. In this plot, the box represents the middle 50% of the data, the end of the upper whisker represents the 75th percentile and the end of the lower whisker represents the 25th percentile of the populations. The median is represented by the line inside of the data box. The statistical outliers of each data set are represented by circles outside of the whisker area. Only expression of N-Ras targeted to the inner and outer mitochondrial compartments (COX-N-Ras: N-Ras-OMP) restored wild-type levels of mitochondrial size.

3.7. N-Ras knockout cells demonstrate increased mitochondrial stress

Mitochondria communicate with the nucleus through a process known as retrograde signaling (or retrograde response). This retrograde signaling contributes to the coordinated expression of nuclear and mitochondrial genes that are essential for mitochondrial functions, such as those in the electron transport chain (ETC). Retrograde signaling has also been recently implicated in communicating mitochondrial instability, such as in cases where mutations result in compromising the efficiency of the ETC. Retrograde signaling results in increased gene expression of glycolytic enzymes in an effort to compensate for a less efficient respiratory process [22]. Several reports demonstrate that cells respond to increased mitochondrial stress through increased activity of both CHOP (GADD153) and NFκB transcription factors [22,29,33,54–56]. The current paradigm suggests that signaling (retrograde) from the mitochondria to the nucleus results in altered nuclear gene expression to protect cells from enhanced mitochondrial stress. We tested, therefore, whether N-Ras negative cells possessed increased basal CHOP or NFκB activity. Cells were transiently transfected with these respective luciferase reporter constructs along with a renilla luciferase construct for normalization. Since the N-Ras negative cells undergo some degree of apoptosis upon reaching confluency (Wolfman et al, unpublished observation), great care was taken so that all cultures were never greater than 50% confluent at the time of harvest. We have previously shown that basal expression of MMP-2 is independent of N-Ras expression, relying solely on the expression of c-K(B)-Ras [5]. Therefore, the MMP-2 luciferase reporter construct was used as a negative control for these initial experiments. As expected, the level of MMP-2 promoter activity was independent of N-Ras expression. This was not the case for either NFκB or CHOP transcriptional activity. Both NFκB and CHOP promoters were significantly more active in the absence of c-N-Ras, suggesting that the lack of c-N-Ras might induce mitochondrial stress, resulting in cellular compensation by increased NFκB and CHOP as a protective adaptation (Fig. 7A). As an independent verification, we also performed RT-PCR analysis for CHOP mRNA. These experiments, shown in Fig. 7B, were performed in a semi-quantitative fashion, using 25 cycles of amplification. Equal amounts of cDNA were amplified (see the β-Actin panel) using CHOP specific primers. The RT-PCR analysis is in good agreement with the luciferase assay, demonstrating an elevated basal level of CHOP mRNA in the N-Ras negative cells, though not in the K-Ras negative cells. Both the luciferase and RT-PCR analysis suggest that c-N-Ras provides a steady-state function that down-regulates CHOP transcriptional activity.

3.8. N-Ras localized to the inner mitochondrial membrane abrogates stress response

To determine whether c-N-Ras localized to the mitochondria contributed to the altered CHOP and NFκB activity in the N-Ras negative background, we analyzed CHOP and c-Jun reporter activity in wild-type and N-Ras knockout cells, as well as cells expressing N-Ras in the inner mitochondrial membrane (COX-N-Ras) and the outer mitochondrial membrane (N-Ras-OMP) (Fig. 7C). These data suggest that c-N-Ras localized only to the inner mitochondrial compartment, not the outer, influences mitochondrial-dependent CHOP activity. Similar results were obtained with NFκB activity (data not shown). Fig. 7D shows that both CHOP and NFκB activity is constitutively increased in the N-Ras knockout cells, and that expression of COX-N-Ras abrogates this activity. No effect was observed with either c-Jun or MMP-2 promoter activity. Since N-Ras-SAAX, a mutant that is not post-translationally modified, does localize to the inner mitochondrial compartment, we tested whether N-Ras-SAAX provided protection from mitochondrial stress, similar to COX-N-Ras localized through its N-terminally fused targeting sequence. CHOP activity was compared in cells ectopically expressing either COX-N-Ras or N-Ras-SAAX. The data shown in Fig. 7E reveal that N-Ras-SAAX is as effective as COX-N-Ras at reducing the elevated basal CHOP activity. This is the first demonstration for a farnesylation independent Ras function.

3.9. N-Ras knockout cells fail to respond to induced mitochondrial stress

The above data suggest that cells lacking c-N-Ras possess a constitutively elevated level of mitochondrial stress, which is abrogated by the presence of c-N-Ras in the inner mitochondrial membrane. To extend these results, we studied the response of these cells to an induced form of mitochondrial stress. Others have reported an increase in CHOP transcriptional activity by inducing an "unfolded protein response" within mitochondria. This is achieved by transiently expressing a deletion mutant of OTC (ornithine transcarbamylase: ΔOTC) that does not refold properly once trafficked to the inner mitochondrial compartment [29]. In two separate experiments, described in Fig. 8A, ΔOTC or control plasmid were transiently transfected along with the CHOP reporter construct into either wild-type (N+/+) or N-Ras knockout (N−/−) cells. CHOP reporter activity was normalized to the level of renilla activity and expressed as the level of CHOP induction (ΔOTC/control). The data demonstrate that the N+/+ cells respond to ΔOTC expression as predicted, with increased CHOP transcriptional activity, four-fold in one experiment and 10 fold in its repeat. In both experiments, the level of CHOP activity induced by ΔOTC in

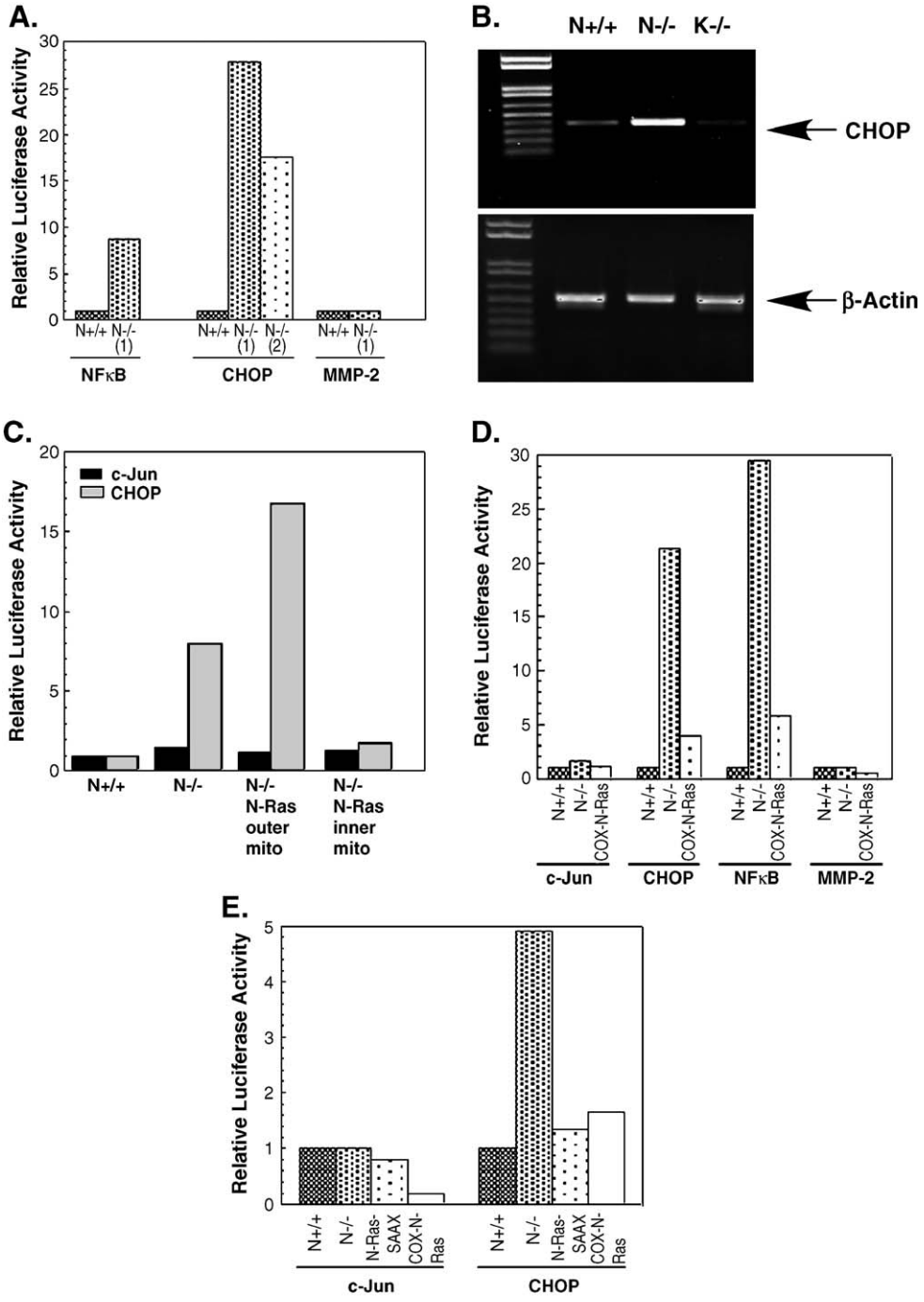


Fig. 7. N-Ras down-regulates constitutive CHOP activity. (A) Wild-type (N+/+) and N-Ras knockout (N−/−) cells were transiently transfected with the indicated luciferase reporter constructs as described in Materials and methods. Activity of the reporter constructs was assessed using the Promega Dual-luciferase kit as described by the manufacturer. N−/−(1) and N−/−(2) represent two independently immortalized N-Ras negative cell lines. The data are averages of triplicate samples and are representative of at least three separate determinations. (B) mRNA isolated from wild-type (N+/+), N-Ras knockout (N−/−) and K-Ras knockout (K−/−) cells were analyzed for the presence of CHOP mRNA by RT-PCR analysis. mRNA levels of β-actin acted as a control for the amplification of equal amounts of cDNA. (C) Wild-type (N+/+), N-Ras knockout (N−/−), and N-Ras knockout cell lines restored with inner mitochondrial membrane targeted N-Ras (COX-N-RAS) or outer mitochondrial membrane targeted N-Ras (N-RAS-OMP) were assessed for CHOP promoter activity using transiently transfected cells and the Promega Dual-luciferase kit as described in A. The data are averages of triplicate samples and are representative of at least three separate determinations. (D) Wild-type (N+/+), N-Ras knockout (N−/−), and N-Ras knockout cell lines restored with inner mitochondrial membrane targeted N-Ras (COX-N-RAS) were assessed for c-jun, CHOP, NFκB and MMP-2 promoter activity using transiently transfected cells and the Promega Dual-luciferase kit. The data are averages of triplicate samples and are representative of at least three separate determinations. (E) Wild-type (N+/+), N-Ras knockout (N−/−), and N-Ras knockout cell lines restored with inner mitochondrial membrane targeted N-Ras (COX-N-RAS) or N-Ras-SAAX (N-Ras-SAAX) were assessed for c-Jun and CHOP transcriptional activity using transiently transfected cells and the Promega Dual-luciferase kit. The data are averages of triplicate samples and are representative of at least three separate determinations.

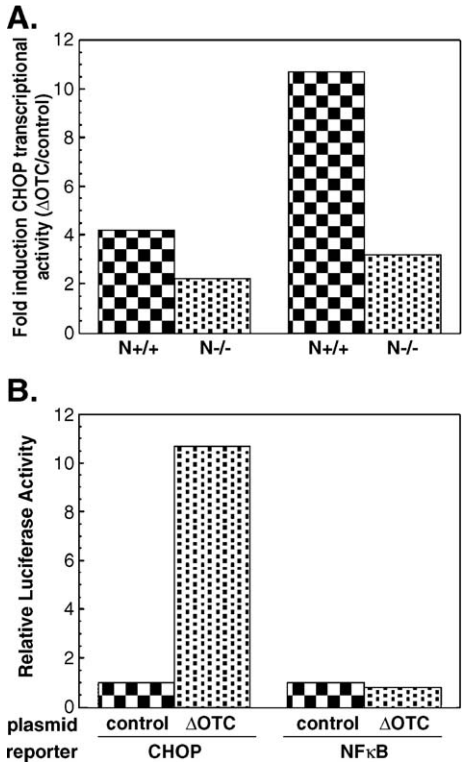


Fig. 8. N-Ras regulates mitochondrial stress responses. (A) Wild-type (N+/+) and N-Ras knockout (N−/−) cells were transiently transfected with ΔOTC or an empty vector and assayed for CHOP transcriptional activity using the Promega Dual-luciferase kit. The graph represents fold transcriptional activity in two separate experiments, each performed in triplicate. (B) Wildtype (N+/+) cells were transiently transfected with ΔOTC or an empty vector and assayed for CHOP and NF-κB transcriptional activity using the Promega Dual-luciferase kit.

the N-Ras negative background was significantly below the level observed in the N+/+ background. These data suggest that the elevated basal CHOP activity observed in the N-Ras negative background is likely to arise from the same mechanism that upregulates CHOP activity in the presence of ΔOTC expression. Surprisingly, challenging wild-type cells with ΔOTC did not elicit an increase in NFκB reporter activity (Fig. 8B), suggesting the possibility that mitochondrial N-Ras might regulate two distinct 'retrograde signaling' pathways.

4. Discussion

The data presented in this report suggest that c-N-Ras is constitutively localized to the inner and outer mitochondrial compartments. The susceptibility of a portion of the mitochondrial N-Ras pool to protease digestion implies that the portion that co-isolates with outer mitochondrial membranes likely faces the cytosol and is attached to the outer mitochondrial membrane through its C-terminal lipid modifications. The identification of a second mitochondrial N-Ras pool, in the inner compartment, suggests two distinct targeting mechanisms, since nuclear encoded proteins destined for the inner mitochondrial compartment require presentation to the TIM/TOM protein translocase complexes for unfolding, transport into the mitochondria and then refolding once they reach their final sub-mitochondrial destination [57,58]. The targeting of c-N-Ras to its two mitochondrial compartments is unlikely to be determined solely by c-N-Ras posttranslational modifications, since c-K(A)-Ras is posttranslationally modified almost identically to c-N-Ras and is not localized to the mitochondria. The only difference in their posttranslational modifications is the position of the palmitate group, with this group being a single amino acid further away from the farnesyl group in c-K(A)-Ras than in c-N-Ras [3]. Additionally, N-Ras-SAAX, which is not post-translationally modified, localizes to the inner mitochondrial compartment, further suggesting a novel c-N-Ras targeting mechanism.

To determine whether c-N-Ras expression directly impacted on mitochondrial function, we examined the ultrastructure of mitochondrial in the N-Ras negative background. The vast majority of the mitochondria in these cells were abnormally shaped, being very dense with tightly packed concentric cristae. Ectopic expression of c-N-Ras restored relatively normal mitochondrial appearance and cristae structure. This only implies that c-N-Ras, not necessarily mitochondrial localized c-N-Ras, contributes to normal mitochondrial structure. We then targeted ectopic c-N-Ras expression to either the inner or outer mitochondrial compartments. Neither c-N-Ras targeted expression to either the inner or outer mitochondrial compartments was sufficient by themselves to restore normal mitochondrial morphology. Only co-expression of c-N-Ras targeted to both mitochondrial compartments had any significant effect on restoring normal mitochondrial structure. These data demonstrate that (1) c-N-Ras expression is critically important to the maintenance of normal mitochondrial structure and (2) c-N-Ras localized to both the inner and outer mitochondrial compartments are likely to provide distinct, non-overlapping functions that impact on mitochondrial structure and function.

K-Ras negative cells also contain mitochondria with an abnormal morphology. Ectopic expression of c-K(B)-Ras, not c-K(A)-Ras restored normal mitochondrial morphology. This observation is consistent with the data shown in Fig. 2 demonstrating that c-K(B)-Ras not c-K(A)-Ras is detected in purified mitochondrial preparations. Overall, the data presented in this report support the idea that both c-N-Ras and c-K(B)-Ras are constitutively associated with intact mitochondrial structures and contribute functions that impinge on normal mitochondrial morphology. The data regarding c-K(B)-Ras are in good agreement with a recent publication by Bivona et al. This group described the PKC-dependent serine 181 phosphorylation dependent translocation of K(B)-Ras from the plasma membrane to the outer mitochondrial membrane [45]. We have confirmed the outer mitochondrial membrane localization of c-K(B)-Ras in untreated fibroblast cultures, suggesting that a portion of the total c-K(B)-Ras pool is phosphorylated on serine 181 under steady-state conditions. The abnormal mitochondrial morphology in the K-Ras negative background that is restored by ectopic expression of c-K(B)-Ras supports a steady-state role for c-K(B)-Ras in mitochondrial function.

Based on the localization of cellular Ras isoforms to intact mitochondria, we tested whether their absence might influence retrograde signaling. N-Ras negative cells, not K-Ras negative cells (data not shown), possessed elevated levels of basal CHOP

and NFκB transcriptional activity, suggesting a potential role in the retrograde signaling process. This was confirmed by RT-PCR analysis of the CHOP mRNA. CHOP and NFκB transcriptional activity was restored to basal levels by the (1) ectopic expression of c-N-Ras (data not shown) and (2) targeted expression of c-N-Ras to the inner mitochondrial compartment, but not the outer mitochondrial membrane. Unlike mitochondrial morphology, CHOP and NFκB transcriptional activity appear to depend only on N-Ras within the inner mitochondrial compartment. N-Ras negative cells were unable to generate a robust CHOP transcriptional response to the induction of mitochondrial stress, using ΔOTC, as the control cells. This suggests that in the absence of c-N-Ras, the mitochondrial-dependent activation of CHOP transcriptional activity is nearly 'maxed out'. This implies that c-N-Ras localized to the inner mitochondrial compartment is likely to regulate the retrograde signaling response. Interestingly, induction of mitochondrial stress using ΔOTC did not elicit any increase in NFκB transcriptional activity, suggesting that c-N-Ras regulates two separate arms of the retrograde response, only one of which is activated by ΔOTC expression.

Ras protein function has always been associated with its ordered C-terminal posttranslational modifications. The C-terminal CAAX motif, common to all Ras isoforms, is recognized by farnesyltransferase that deposits a farnesyl moiety on the Cys residue. Following this initial posttranslational modification, the C-terminal 3 amino acids are removed, the Cys is further methylated and palmitate is added to Cys residues just N-terminal to the farnesylated Cys residue. This later palmitoylation, through a palmitoyl transferase, assists in stabilizing the interaction between farnesylated Ras and membrane compartments. Replacing the terminal Cys residue with Ser results in an unfarnesylated Ras protein that is soluble and acts as a dominant negative inhibitor of traditional Ras-dependent signal transduction [59–61]. The localization of N-Ras-SAAX to the inner mitochondrial compartment and its ability to reduce the constitutively elevated CHOP activity to control levels, similar to ectopic expression of c-N-Ras and COX-N-Ras suggests that N-Ras-SAAX is functionally active within the inner mitochondrial compartment. This is the first demonstration of a farnesylation independent Ras function. This data also implies that the targeting of c-N-Ras to the inner mitochondrial compartment is most likely to depend on the C-terminal hypervariable sequence upstream of the CAAX motif.

The identification of Ras isoform specific subcellular distributions within endomembrane structures provides new implications to the expression of oncogenic Ras isoforms. The apparent redundancy of Ras isoform function in acute signal transduction is likely explained by the potential of Ras isoforms to regulate exchange factor activity [62–65]. However, expression of oncogenic K(B)-Ras versus N-Ras will have clearly different consequences on mitochondrial function. These differences might be valuable points for tumor specific therapeutic intervention. This was clearly demonstrated by Bivona et al. In their system, PKC-dependent translocation of c-K(B)-Ras to the outer mitochondrial membrane was pro-apoptotic. They extended this model into G12V-K(B)-Ras dependent tumors. Treating mice bearing these tumors with agents that increased PKC activation did result in decreased tumor size, presumably through translocation of G12V-K(B)-Ras to the mitochondria thereby increasing its pro-apoptotic affects [45]. Clearly, the consequences of c-N-Ras function within the mitochondria are more complex than that of c-K(B)-Ras. It is unclear whether there is a direct cooperative relationship between the two mitochondrial c-N-Ras pools, or whether their independent functions both contribute to normal mitochondrial morphology. Our data indicate that at least one function of c-N-Ras within the inner mitochondrial compartment is independent of its outer mitochondrial pool. c-N-Ras within the inner mitochondrial compartment is able to affect the transcriptional activity of both CHOP and NFκB in the absence of c-N-Ras in the outer mitochondrial membrane. However, this does not directly restore normal mitochondrial morphology. Clearly, future work will focus on the mechanism(s) through which c-N-Ras in the inner mitochondrial compartment transmits its signal through the inner mitochondrial space and outer mitochondrial membrane to the nucleus and affect the levels of CHOP mRNA and NFκB transcriptional activity. We will also determine whether the ability of c-N-Ras within the inner mitochondrial compartment to regulate retrograde signaling is specific for N-Ras or whether, once targeted to the inner mitochondrial compartment, any Ras isoform will suffice.

Acknowledgements

This work was partially supported by NIH grant number GM626466. The authors would like to thank Dr. Tom Patterson, Dr. Andrew Larner and Dr. Joe DiDonato for their helpful discussions.

References

[1] S.L. Campbell, R. Khosravi-Far, K.L. Rossman, G.J. Clark, C.J. Der, Increasing complexity of Ras signaling, Oncogene 17 (1998) 1395–1413.
[2] S.A. Moodie, A. Wolfman, The 3Rs of life: Ras, Raf and growth regulation, Trends Genet. 10 (1994) 44–48.
[3] M. Barbacid, Ras genes, Annu. Rev. Biochem. 56 (1987) 779–827.
[4] R.J.A. Grand, D. Owen, The biochemistry of Ras p21, Biochem. J. 279 (1991) 609–631.
[5] J. Liao, J.C. Wolfman, A. Wolfman, K-Ras regulates the steady-state expression of matrix metalloproteinase 2 in fibroblasts, J. Biol. Chem. 278 (2003) 31871–31878.
[6] J.C. Wolfman, A. Wolfman, Endogenous c-N-Ras provides a steady-state anti-apoptotic signal, J. Biol. Chem. 275 (2000) 19315–19323.
[7] A. Rebollo, D. Perez-Sala, A.C. Martinez, Bcl-2 differentially targets K-, N-, and H-Ras to mitochondria in IL-2 supplemented or deprived cells: Implications in prevention of apoptosis, Oncogene 18 (1999) 4930–4939.
[8] T.G. Bivona, M.R. Philips, Ras pathway signaling on endomembranes, Curr. Opin. Cell Biol. 15 (2003) 136–142.
[9] M.J. Caloca, J.L. Zugaza, X.R. Bustelo, Exchange factors of the RasGRP family mediate Ras activation in the Golgi, J. Biol. Chem. (2003) 33465–33473.
[10] V.K. Chiu, T. Bivona, A. Hach, J.B. Sajous, J. Silletti, H. Wiener, R.L. Johnson II, A.D. Cox, M.R. Philips, Ras signalling on the endoplasmic reticulum and the Golgi, Nat. Cell Biol. 2 (2002) 2.
[11] E. Choy, V.K. Chiu, J. Silletti, M. Feoktistov, T. Morimoto, D. Michaelson, I.E. Ivanov, M.R. Philips, Endomembrane trafficking of Ras: the CAAX motif targets proteins to the ER and Golgi [see comments], Cell 98 (1999) 69–80.

[12] M.R. Philips, Compartmentalized signalling of Ras, Biochem. Soc. Trans. 33 (2005) 657–661.
[13] M. Roudebush, T. Slabe, V. Sundaram, C.L. Hoppel, M. Golubic, D.W. Stacey, Neurofibromin colocalizes with mitochondria in cultured cells, Exp. Cell Res. 236 (1997) 161–172.
[14] H.G. Wang, U.R. Rapp, J.C. Reed, Bcl-2 targets the protein kinase Raf-1 to mitochondria, Cell 87 (1996) 629–638.
[15] H.G. Wang, J.C. Reed, Bc1-2, Raf-1 and mitochondrial regulation of apoptosis, BioFactors 8 (1998) 13–16.
[16] P. Salomoni, M.A. Wasik, R.F. Riedel, K. Reiss, J.K. Choi, T. Skorski, B. Calabretta, Expression of constitutively active Raf-1 in the mitochondria restores antiapoptotic and leukemogenic potential of a transformation-deficient BCR/ABL mutant, J. Exp. Med. 187 (1998) 1995–2007.
[17] I.A. Prior, J.F. Hancock, Compartmentalization of Ras proteins, J. Cell Sci. 114 (2001) 1603–1608.
[18] I. Arozarena, D. Matallanas, M.T. Berciano, V. Sanz-Moreno, F. Calvo, M.T. Munoz, G. Egea, M. Lafarga, P. Crespo, Activation of H-Ras in the endoplasmic reticulum by the RasGRF family guanine nucleotide exchange factors, Mol. Cell. Biol. 24 (2004) 1516–1530.
[19] S. Goffart, R.J. Wiesner, Regulation and co-ordination of nuclear gene expression during mitochondrial biogenesis, Exp. Physiol. 88 (2003) 33–40.
[20] A.M. Kroon, C. Van den Bogert, Biogenesis of mitochondria and genetics of mitochondrial defects, J. Inherit. Metab. Dis. 10 (Suppl. 1) (1987) 54–61.
[21] G. Attardi, G. Schatz, Biogenesis of mitochondria, Annu. Rev. Cell Biol. 4 (1988) 289–333.
[22] R.A. Butow, N.G. Avadhani, Mitochondrial signaling: the retrograde response, Mol. Cell 14 (2004) 1–15.
[23] T. Sekito, J. Thornton, R.A. Butow, Mitochondria-to-nuclear signaling is regulated by the subcellular localization of the transcription factors Rtg1p and Rtg3p, Mol. Biol. Cell 11 (2000) 2103–2115.
[24] B.A. Rothermel, J.L. Thornton, R.A. Butow, Rtg3p, a basic helix–loop–helix/leucine zipper protein that functions in mitochondrial-induced changes in gene expression, contains independent activation domains, J. Biol. Chem. 272 (1997) 19801–19807.
[25] Z. Liu, T. Sekito, M. Spirek, J. Thornton, R.A. Butow, Retrograde signaling is regulated by the dynamic interaction between Rtg2p and Mks1p, Mol. Cell 12 (2003) 401–411.
[26] I. Dilova, S. Aronova, J.C. Chen, T. Powers, Tor signaling and nutrient-based signals converge on Mks1p phosphorylation to regulate expression of Rtg1.Rtg3p-dependent target genes, J. Biol. Chem. 279 (2004) 46527–46535.
[27] A. Chelstowska, R.A. Butow, RTG genes in yeast that function in communication between mitochondria and the nucleus are also required for expression of genes encoding peroxisomal proteins, J. Biol. Chem. 270 (1995) 18141–18146.
[28] P.A. Kirchman, S. Kim, C.Y. Lai, S.M. Jazwinski, Interorganelle signaling is a determinant of longevity in *Saccharomyces cerevisiae*, Genetics 152 (1999) 179–190.
[29] Q. Zhao, J. Wang, I.V. Levichkin, S. Stasinopoulos, M.T. Ryan, N.J. Hoogenraad, A mitochondrial specific stress response in mammalian cells, EMBO J. 21 (2002) 4411–4419.
[30] T. Arnould, S. Vankoningsloo, P. Renard, A. Houbion, N. Ninane, C. Demazy, J. Remacle, M. Raes, CREB activation induced by mitochondrial dysfunction is a new signaling pathway that impairs cell proliferation, EMBO J. 21 (2002) 53–63.
[31] G. Amuthan, G. Biswas, S.Y. Zhang, A. Klein-Szanto, C. Vijayasarathy, N.G. Avadhani, Mitochondria-to-nucleus stress signaling induces phenotypic changes, tumor progression and cell invasion, EMBO J. 20 (2001) 1910–1920.
[32] C. Vijayasarathy, S. Damle, S.K. Prabu, C.M. Otto, N.G. Avadhani, Adaptive changes in the expression of nuclear and mitochondrial encoded subunits of cytochrome c oxidase and the catalytic activity during hypoxia, Eur. J. Biochem. 270 (2003) 871–879.
[33] G. Biswas, O.A. Adebanjo, B.D. Freedman, H.K. Anandatheerthavarada, C. Vijayasarathy, M. Zaidi, M. Kotlikoff, N.G. Avadhani, Retrograde Ca2 + signaling in C2C12 skeletal myocytes in response to mitochondrial genetic and metabolic stress: a novel mode of inter-organelle crosstalk, EMBO J. 18 (1999) 522–533.
[34] J. St-Pierre, J. Lin, S. Krauss, P.T. Tarr, R. Yang, C.B. Newgard, B.M. Spiegelman, Bioenergetic analysis of peroxisome proliferator-activated receptor gamma coactivators 1alpha and 1beta (PGC-1alpha and PGC-1beta) in muscle cells, J. Biol. Chem. 278 (2003) 26597–26603.
[35] S. Salvioli, M. Bonafe, M. Capri, D. Monti, C. Franceschi, Mitochondria, aging and longevity—a new perspective, FEBS Lett. 492 (2001) 9–13.
[36] H. Umanoff, W. Edelmann, A. Pellicer, R. Kucherlapati, The murine N-Ras gene is not essential for growth and development, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 1709–1713.
[37] L. Johnson, D. Greenbaum, K. Cichowski, K. Mercer, E. Murphy, E. Schmitt, R.T. Bronson, H. Umanoff, W. Edelmann, R. Kucherlapati, T. Jacks, K-Ras is an essential gene in the mouse with partial functional overlap with N-Ras, Genes Dev. 11 (1997) 2468–2481.
[38] G.J. Todaro, H. Green, Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines, J. Cell Biol. 17 (1963) 299–313.
[39] F. Gasnier, R. Rousson, F. Lerme, E. Vaganay, P. Louisot, O. Gateau-Roesch, Use of Percoll gradients for isolation of human placenta mitochondria suitable for investigating outer membrane proteins, Anal. Biochem. 212 (1993) 173–178.
[40] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[41] Y. Nemoto, P. De Camilli, Recruitment of an alternatively spliced form of synaptojanin 2 to mitochondria by the interaction with the PDZ domain of a mitochondrial outer membrane protein, EMBO J. 18 (1999) 2991–3006.
[42] J.V. Fleming, N. Fontanier, D.N. Harries, W.D. Rees, The growth arrest genes gas5, gas6, and CHOP-10 (gadd153) are expressed in the mouse preimplantation embryo, Mol. Reprod. Dev. 48 (1997) 310–316.
[43] M. Hamilton, J. Liao, M.K. Cathcart, A. Wolfman, Constitutive association of c-N-Ras with c-Raf-1 and protein kinase cepsilon in latent signaling modules, J. Biol. Chem. 276 (2001) 29079–29090.
[44] J.C. Wolfman, T. Palmby, C.J. Der, A. Wolfman, Cellular N-Ras promotes cell survival by downregulation of jun N-terminal protein kinase and p38, Mol. Cell. Biol. 22 (2002) 1589–1606.
[45] T.G. Bivona, S.E. Quatela, B.O. Bodemann, I.M. Ahearn, M.J. Soskis, A. Mor, J. Miura, H.H. Wiener, L. Wright, S.G. Saba, D. Yim, A. Fein, I. Perez de Castro, C. Li, C.B. Thompson, A.D. Cox, M.R. Philips, PKC regulates a farnesyl-electrostatic switch on K-Ras that promotes its association with Bcl-Xl on mitochondria and induces apoptosis, Mol. Cell 21 (2006) 481–493.
[46] G.D. Malloch, L.A. Munday, M.S. Olson, J.B. Clark, Comparative development of the pyruvate dehydrogenase complex and citrate synthase in rat brain mitochondria, Biochem. J. 238 (1986) 729–736.
[47] E. Leshinsky-Silver, A. Zinger, C.N. Bibi, V. Barash, M. Sadeh, D. Lev, T.L. Sagie, MEHMO (Mental retardation, Epileptic seizures, Hypogenitalism, Microcephaly, Obesity): a new X-linked mitochondrial disorder, Eur. J. Hum. Genet. 10 (2002) 226–230.
[48] B. Schwartzkopff, H. Frenzel, G. Breithardt, M. Deckert, B. Losse, K.V. Toyka, M. Borggrefe, W. Hort, Ultrastructural findings in endomyocardial biopsy of patients with Kearns–Sayre syndrome, J. Am. Coll. Cardiol. 12 (1988) 1522–1528.
[49] D.W. Walker, S. Benzer, Mitochondrial "swirls" induced by oxygen stress and in the Drosophila mutant hyperswirl, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 10290–10295.
[50] G.B. John, Y. Shang, L. Li, C. Renken, C.A. Mannella, J.M. Selker, L. Rangell, M.J. Bennett, J. Zha, The mitochondrial inner membrane protein mitofilin controls cristae morphology, Mol. Biol. Cell 16 (2005) 1543–1554.
[51] D. Matallanas, I. Arozarena, M.T. Berciano, D.S. Aaronson, A. Pellicer, M. Lafarga, P. Crespo, Differences on the inhibitory specificities of H-Ras, K-Ras and N-Ras (N17) dominant negative mutants are related to their membrane microlocalization, J. Biol. Chem. (2002) 4572–4581.
[52] J.J. Fiordalisi, S.P. Holly, R.L. Johnson II, L.V. Parise, A.D. Cox, A distinct class of dominant negative Ras mutants: cytosolic GTP-bound Ras

effector domain mutants that inhibit Ras signaling and transformation and enhance cell adhesion, J. Biol. Chem. 277 (2002) 10813–10823.
[53] C. Horie, H. Suzuki, M. Sakaguchi, K. Mihara, Characterization of signal that directs C-tail-anchored proteins to mammalian mitochondrial outer membrane, Mol. Biol. Cell 13 (2002) 1615–1625.
[54] G. Biswas, H.K. Anandatheerthavarada, M. Zaidi, N.G. Avadhani, Mitochondria to nucleus stress signaling: a distinctive mechanism of NFkappaB/Rel activation through calcineurin-mediated inactivation of IkappaBbeta, J. Cell Biol. 161 (2003) 507–519.
[55] G. Biswas, H.K. Anandatheerthavarada, N.G. Avadhani, Mechanism of mitochondrial stress-induced resistance to apoptosis in mitochondrial DNA-depleted C2C12 myocytes, Cell Death Differ. 12 (2005) 266–278.
[56] G. Biswas, M. Guha, N.G. Avadhani, Mitochondria-to-nucleus stress signaling in mammalian cells: nature of nuclear gene targets, transcription regulation, and induced resistance to apoptosis, Gene 354 (2005) 132–139.
[57] W. Neupert, Protein import into mitochondria, Annu. Rev. Biochem. 66 (1997) 863–917.
[58] N.J. Hoogenraad, L.A. Ward, M.T. Ryan, Import and assembly of proteins into mitochondria of mammalian cells, Biochim. Biophys. Acta 1592 (2002) 97–105.
[59] J.S. Goodwin, K.R. Drake, C. Rogers, L. Wright, J. Lippincott-Schwartz, M.R. Philips, A.K. Kenworthy, Depalmitoylated Ras traffics to and from the Golgi complex via a nonvesicular pathway, J. Cell Biol. 170 (2005) 261–272.
[60] S. Roy, S. Plowman, B. Rotblat, I.A. Prior, C. Muncke, S. Grainger, R.G. Parton, Y.I. Henis, Y. Kloog, J.F. Hancock, Individual palmitoyl residues serve distinct roles in H-Ras trafficking, microlocalization, and signaling, Mol. Cell. Biol. 25 (2005) 6722–6733.
[61] S.G. Coats, M.A. Booden, J.E. Buss, Transient palmitoylation supports H-Ras membrane binding but only partial biological activity, Biochemistry 38 (1999) 12926–12934.
[62] S.M. Margarit, H. Sondermann, B.E. Hall, B. Nagar, A. Hoelz, M. Pirruccello, D. Bar-Sagi, J. Kuriyan, Structural evidence for feedback activation by Ras.GTP of the Ras-specific nucleotide exchange factor SOS, Cell 112 (2003) 685–695.
[63] N.P. Fam, W.T. Fan, Z. Wang, L.J. Zhang, H. Chen, M.F. Moran, Cloning and characterization of Ras-GRF2, a novel guanine nucleotide exchange factor for Ras, Mol. Cell. Biol. 17 (1997) 1396–1406.
[64] R. Zippel, M. Balestrini, M. Lomazzi, E. Sturani, Calcium and calmodulin are essential for Ras-GRF1-mediated activation of the Ras pathway by lysophosphatidic acid, Exp. Cell Res. 258 (2000) 403–408.
[65] P. Villalonga, C. Lopez-Alcala, M. Bosch, A. Chiloeches, N. Rocamora, J. Gil, R. Marais, C.J. Marshall, O. Bachs, N. Agell, Calmodulin binds to K-Ras, but not to H-or N-Ras, and modulates its downstream signaling, Mol. Cell. Biol. 21 (2001) 7345–7354.