Prohibitin, an antiproliferative protein, is localized to mitochondria

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Abstract Prohibitin is a ubiquitously expressed protein with antiproliferative properties. When rat prohibitin tagged with a carboxy-terminal c-Myc epitope was expressed in baby hamster kidney cells the protein was targeted to mitochondria. In immunofluorescence microscopy prohibitin colocalized with a mitochondrial marker E3. Immunoelectron microscopy revealed that prohibitin was associated with the periphery of mitochondria. The amino-terminus of prohibitin shares characteristics of the known mitochondrial import signals, and positioning of the tag at the N-terminus causes accumulation of the protein in the cytoplasm. These findings help to direct functional studies on prohibitin and suggest that a mitochondrial protein may act as a tumor suppressor.

Key words: Prohibitin; Mitochondria; Antiproliferative; Carsinogenesis

1. Introduction

The prohibitin gene was isolated for its ability to negatively regulate cell proliferation. McClung et al. [1] cloned it as one of the cDNAs derived from mRNAs which were more highly expressed in nondividing than regenerating rat liver cells. The antiproliferative activity of prohibitin could be demonstrated by microinjection of the synthetic mRNA into normal fibroblasts and HeLa cells where it blocked the entry of cells into S phase [2]. Prohibitin mRNA expression levels of cultured fibroblasts were found to vary 3- to 4-fold with culture conditions and cell cycle state [3]. However, the mRNA was expressed at roughly comparable levels in all tested rat tissues [2].

The mRNA codes for a protein of 272 amino acids. No protein motifs typical for signal sequences, nuclear localization signals, ATP-binding sites or transcription factors could be identified in the primary structure. A short, 12 residue stretch at the N-terminus was the longest hydrophobic region found in the protein. When the mRNA was translated in vitro it produced a polypeptide of the expected length of about 30 kDa, but this was not translocated into microsomes. Consequently, the in vitro translated protein was not modified by sugar addition or signal peptide cleavage [2]. In cultured cells, the prohibitin protein was found not to reflect directly the variable amounts of mRNA at different stages of the cell cycle, but was rather constitutively expressed with 2- to 3-fold higher levels in rapidly dividing than senescent cells [4]. We have identified a protein of similar molecular weight and isoelectric point in a detergent insoluble cellular membrane fraction from dog kidney epithelial cells. Two tryptic peptide sequences (ILFRPVASQLPR and IYTSIGEDY) obtained from the protein purified from two- dimensional gels [5, protein A26] matched with the published rat sequence and confirmed this protein to be prohibitin.

Prohibitin was found to share substantial homology with a *Drosophila* protein, Cc, of unknown function but vital for normal development and differentiation [2]. Flies homozygous for nonfunctional Cc alleles die during the larva to pupa metamorphosis [6]. The protein seems to be well conserved, as the cloning of the human prohibitin gene revealed only a single amino acid difference to the rat sequence [7]. Interestingly, the human gene maps to chromosome 17q21, a region commonly deleted in breast tumors [8]. Moreover, in a substantial fraction of sporadic human breast cancers showing loss of heterozygosity on the long arm of chromosome 17 or developed in patients 35 years old or younger mutations in the prohibitin gene were identified [7], strengthening the argument of the antiproliferative or tumor suppressive nature of prohibitin. The mechanism by which the antiproliferative activity of prohibitin is mediated remains, however, unknown. As one of the first steps towards understanding the function of prohibitin we investigated its subcellular localization.

2. Materials and methods

2.1. Construction of the tagged prohibitin cDNAs

To generate a construct with an N-terminal c-Myc epitope EQKLISEEDL [9], the wild type rat prohibitin cDNA in the Bluescript plasmid [2] was amplified by PCR with the following oligonucleotides: the 5′ mutagenic primer (5′CAGGTGGATCCATGGAACAAAAACTCATCTCAGAAGAGGATCTGATGGCTGCCAAAGTGTTTGAGTCC3′) encoding the amino acids MEQKLISEEDL preceded by a *Bam*HI site and followed by the first eight amino acids of prohibitin, and the 3′ primer (5′ACGAGGAAGCTGGCTGGCCACCGGCCGGAA3′). The PCR product was digested with *Bam*HI and *Eag*I and ligated into *Eag*I–*Hin*dIII cut prohibitin providing the rest of the coding region and *Bam*HI–*Hin*dIII cut pBluescript . Thus, the 5′ end of the construct containing the tag just upstream the initiator methionine faces the T7 promoter of the plasmid. To add the c-Myc tag to the C-terminus the prohibitin cDNA was amplified with the 5′ primer (5′GAAGCAGAGAGAGCCAGATTTGTGGTGGAA3′) and the 3′ mutagenic primer encoding the amino acids EQKLISEEDL after the last amino acid of prohibitin and a *Hin*dIII cloning site (5′CGATCCGAAGCTTTTACAGATCCTCTTCTGAGATGAGTTTTTGTTCCTGGGGGAGCTGGAGGAGCAC3′). The PCR product digested with *Xcm*I and *Hin*dIII was ligated into *Bam*HI–*Xcm*I cut prohibitin and *Bam*HI–*Hin*dIII cut pBluescript. All the regions generated by PCR were confirmed by sequencing to be correct.

2.2. Cell culture and transfection

BHK 21 cells were grown in Glasgow's modified Eagle's medium supplemented with 5% FCS, 10% tryptose phosphate broth, 2 mM glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin. The cells grown on cover slips were 80% confluent on the day of transfection. The cells were washed with serum-free medium and infected with T7 RNA

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Abbreviations: cDNA, complementrary DNA; PCR, polymerase chain reaction; BHK, baby hamster kidney; FCS, fetal calf serum; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

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polymerase-recombinant vaccinia virus [10] using 3–5 pfu/cell. The infection was carried out at room temperature for 30 min, after which the cells were transfected with the 5′ or 3′ myc-tagged prohibitin plasmids using DOTAP reagent (Bœhringer Mannheim GmbH) according to the manufacturer's instructions. After 4 h incubation at 37°C 5% CO_2, cycloheximide (20 μg/ml) was added to the cells and the expression continued for additional 1.5–2 h before fixation.

2.3. Immunofluorescence microscopy

The cells were fixed with 4% paraformaldehyde in PBS for 20 min and after rinsing with PBS incubated in ice-cold methanol for 5 min at −20 °C. After fixation the cells were washed with PBS and the aldehyde groups quenched with 50 mM NH_4Cl in PBS for 15 min followed by PBS wash. The cells were permeabilized with 0.1% Triton X-100 for 4 min, washed with PBS and unspecific antibody binding was blocked by incubating the cells in 10% heat-inactivated FCS for 30 min. The purified ascites solution of the monoclonal antibody 9E10 against the human c-Myc epitope was diluted 1:1000 and the polyclonal anti-E3 antibody 1:200 in 5% FCS. The mouse cell line producing 9E10 was purchased from the European Collection of Animal Cell Cultures (no. 85102202). The anti-E3 antibody was a kind gift from Dr. Masatoshi Hakozaki (Fukushima Medical College, Fukushima, Japan). The cells were incubated 30 min at room temperature with the primary antibodies, washed effectively with PBS and the primary antibodies visualized with pre-adsorbed tetramethyl rhodamine isothiocyanate-conjugated donkey anti-mouse or fluorescein isothiocyanate-conjugated donkey anti-rabbit antibodies (Dianova, Hamburg, Germany). The cover slips mounted in Moviol were viewed and photographed with an Axiophot photomicroscope (Carl Zeiss, Oberkochen, Germany).

2.4. Immunoelectron microscopy

BHK cells were transfected as described above except that the transfection time was increased to 6.5 h and incubation with cycloheximide omitted to maximize the number of cells transfected. After fixation in 8% paraformaldehyde in 250 mM HEPES, pH 7.35, the cells were scraped from the culture dish, pelleted, and processed for cryosectioning as described previously [11]. Thawed sections were labeled by sequential incubations with the 9E10 culture supernatant, followed by secondary antibody and 10 nm protein A-gold. The distribution of gold particles associated with mitochondrial profiles was ascertained on micrographs taken at a primary magnification of 20 k ×. Gold particles associated with 30 mitochondrial profiles were classified as (a) peripheral (within 30 nm of the periphery of the mitochondria), (b) associated with the cristae (within 30 nm of the membranes of the cristae), (c) within the matrix, and (d) other (position unclear).

2.5. Western blotting

BHK cells transfected with the 5′ or 3′ Myc-tagged prohibitin constructs were lysed in 1% SDS and the proteins separated by reducing 12% SDS-PAGE. The proteins were transferred to nitrocellulose in a

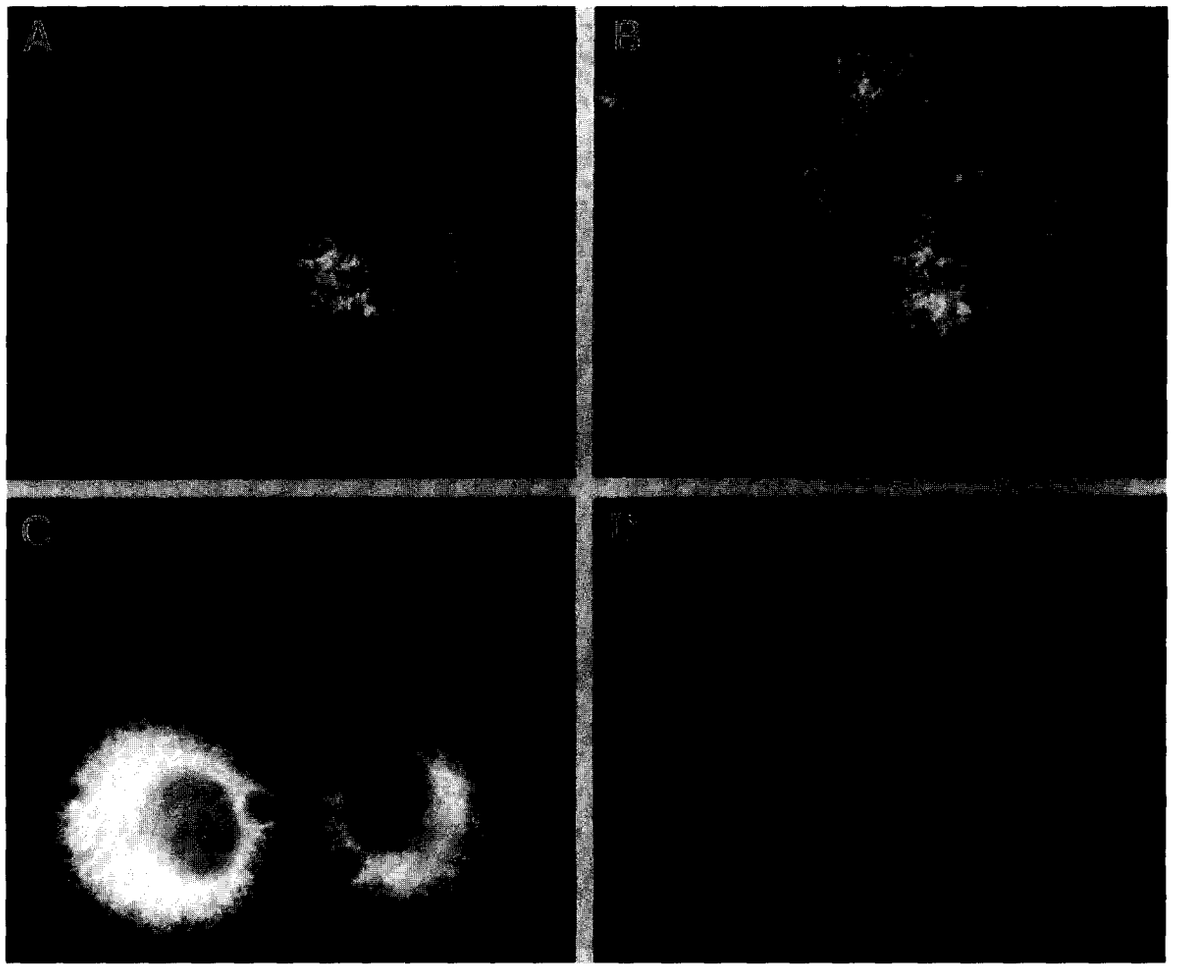

Fig. 1. Immunofluorescence localization of epitope tagged prohibitin. The T7 RNA polymerase - recombinant vaccinia virus expression system was used to express either C- or N-terminally Myc-tagged prohibitin in BHK cells and the overexpressed protein was immunostained using the Myc-antibody. In a cell expressing the C-terminally tagged prohibitin (A) the labeling colocalizes with the mitochondrial marker E3 (B), whereas cells expressing the N-terminally tagged prohibitin (C) show strong cytoplasmic immunoreactivity which does not colocalize with E3 (D). Bars, 10 μm.

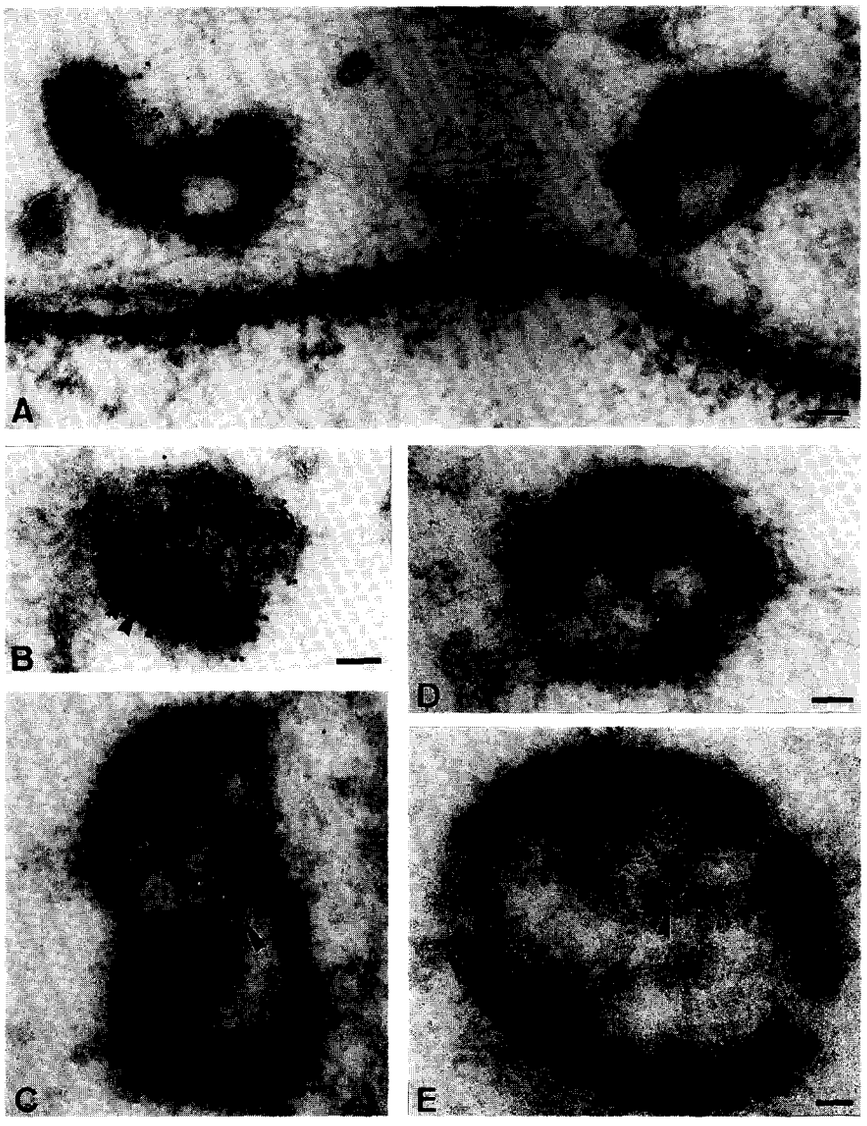

Fig. 2. Immunoelectron microscopy localization of epitope tagged prohibitin. BHK cells were transfected with C-terminally Myc-tagged prohibitin, processed for frozen sectioning and labeled as detailed in section 2. A, Labeling is associated with the peripheral membrane of mitochondria (m) but not with other membranes (e.g. the nuclear envelope, n). B–E, Labeling is predominantly present around the periphery of the mitochondria but some gold label (large arrowheads) is occasionally seen close to the cristae (small arrowheads). Bars, 100 nm.

blotting buffer consisting of 25 mM Tris, 190 mM glycine and 20% methanol. The liter was incubated in a blocking solution (5% non-fat dried milk, 0.1% Tween 20 in PBS) for 1 h at room temperature before binding of the anti-myc 9E10 antibody (diluted 1:5000 in blocking solution) overnight at 4°C. The bands were detected by chemiluminiscence using horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Jackson Immuno Research Inc.) and the ECL reagent (Amersham).

3. Results and discussion

To localize prohibitin intracellularly, we tagged the protein with the c-Myc epitope (EQKLISEEDL) either at the N- or the C-terminus. Two different locations for the tag were used to exclude possible mislocalizations that could be caused by the tag interfering with putative unknown targeting signals. The tagged proteins were overexpressed using the vaccinia T7 system in BHK cells, and visualized by the Myc-antibody. The staining obtained with the C-terminally tagged protein was punctate, in the form of fairly large dots as well as elongated structures, evenly distributed throughout the cytoplasm (Fig. 1A). The shape, from round to tubular, and size of the labeled structures resembled mitochondrial staining. We therefore double-stained the infected BHK cells for prohibitin and E3, dihydrolipoamide dehydrogenase, a component in the mitochondrial 2-oxo acid dehydrogenase complexes (for review see [12]). The labelings colocalized precisely indicating that the C-terminally tagged prohibitin is indeed targeted to mitochondria (Fig. 1A and B). The staining pattern remained identical regardless of the transfection time (3 to 7 h), except that more cells expressing prohibitin were found with increasing time (data not shown).

When the N-terminally tagged prohibitin was expressed

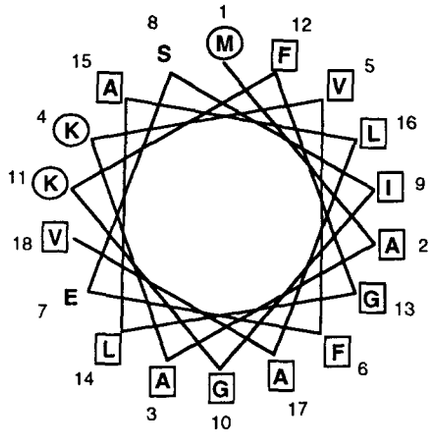

Fig. 3. Amphiphilic helix formed by the N-terminus of prohibitin. Amino-terminal residues 1–18 were plotted on a 'helical wheel' as described by Schiffer and Edmundson [17]. The hydrophobic residues are marked with rectangles and the positively charged ones are encircled.

using the same system the staining pattern was, surprisingly, completely different. The protein was found to accumulate in the cytoplasm and showed no colocalization with the mitochondrial marker E3 (Fig. 1C and D). In preliminary experiments where cells were fixed with paraformaldehyde and permeabilized with Triton X-100, the cytoplasmic staining was weak (not shown). However, fixation of proteins with both paraformaldehyde and methanol prevented the antigen from being washed away during the staining and allowed the detection of the diffuse cytoplasmic accumulation of the antigen.

To extend the immunofluorescence microscopic localization of the C-terminally tagged prohibitin we carried out immunoelectron microscopy with the Myc-antibody on ultrathin frozen sections of transfected BHK cells. The antibody labeled specifically only the mitochondria of prohibitin expressing cells (Fig. 2A). There was no significant labeling of other membranes in transfected cells and there was no mitochondrial labeling in untransfected cells. In the transfected cells the labeling was predominantly present around the periphery of the mitochondria (Fig. 2A–E). When the distribution of gold particles associated with mitochondrial profiles was quantitated (see section 2 for details) 78% of the labeling was found to be peripheral, 15% associated with cristae and only 3% within the mitochondrial matrix. In 4% the position of gold particles remained unclear. The fraction of peripheral labeling may be underestimated as a glancing section across the periphery of a mitochondrion may reveal apparent intramitochondrial labeling. The immunoelectron microscopic localization of prohibitin leaves still open whether the protein is associated with either of the mitochondrial membranes or with the intermembrane space. However, it rules out the possibility that prohibitin would be a matrix protein. Although prohibitin is mostly hydrophilic, membrane association of the protein is not excluded as the N-terminal hydrophobic stretch may be long enough to form a membrane span [13].

Most proteins directed into mitochondria (whether the final location of the protein will be in the mitochondrial matrix, inner membrane or intermembrane space) carry an N-terminal cleavable presequence containing the mitochondrial targeting signal. This signal is short (about 8–30 amino acids) and has a tendency to form an amphiphilic α-helix upon insertion into lipid membranes [14,15]. The unusual properties of an amphiphilic helix result from the uneven distribution of hydrophobic and hydrophilic residues around the helix. The presequence usually contains several positively charged and hydroxylated residues and few, if any, negative charges. The positively charged amino acids and the hydrophobic residues are facing opposite sides of the helix (for review see [16]). As presequences have no obvious primary structure homology it is difficult to predict a mitochondrial localization of a protein from the sequence alone. Interestingly, if the 18 N-terminal residues of prohibitin are plotted on a helical wheel [17] the hydrophobic and uncharged residues are clustered on one side, whereas the positively charged amino acids are on the opposite side (Fig. 3). Moreover, if the c-Myc epitope is inserted at the N-terminus, 3 additional negative charges will be introduced and the amphiphilic helix will be broken. Thus, the sequence analyses agree well with our findings, the N-terminal sequence of the wild type prohibitin favoring mitochondrial import and the N-terminal tag blocking entry.

To visualize the prohibitin polypeptide chains we analyzed the overexpressed proteins from BHK cell lysates by Western blotting with the Myc-antibody. Both tagged constructs gave rise to protein products of the expected size. However, there was a small but reproducible migration difference between the two proteins, the protein tagged at the C-terminus showing a slightly faster mobility on SDS-PAGE than the N-terminally tagged one (Fig. 4). In view of the mitochondrial localization of the C-terminally tagged prohibitin, it is likely that an N-terminal targeting signal is cleaved from the protein following mitochondrial import. However, we cannot exclude the possibility that the position of the tag causes a conformational difference affecting the apparent mobility of the polypeptide chains on SDS-PAGE.

The data unambigously demonstrate that the C-terminally epitope tagged and overexpressed prohibitin is targeted to mitochondria. The possibilities of creating a new artificial C-terminal mitochondrial import signal by the tagging or causing false import into mitochondria due to overexpression seem very unlikely. Furthermore, the mitochondrial localization of prohibitin is in accordance with several previous observations. The protein is constitutively expressed in a wide variety of cell types and the highest protein levels are found in rapidly proliferating cells. The protein is intracellular but is not translocated into

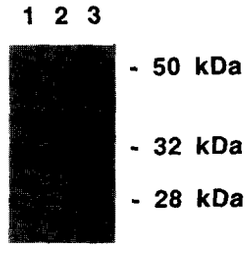

Fig. 4. Immunoblot analysis of prohibitin expression in BHK cells. Proteins from BHK cells expressing either the C-terminally (1) or N-terminally (2) Myc-tagged prohibitin or untransfected cells (3) were probed with the Myc-antibody. The C-terminally tagged prohibitin has a slightly smaller apparent molecular weight than the N-terminally tagged one. A nonspecific band of about 40 kDa is recognized by the antibody in transfected as well as in untransfected cells.

microsomes, nor does it have a clear signal sequence. Hence, prohibitin falls into the same category as most mitochondrial proteins, being synthesized on cytosolic polysomes and transported into the organelle. The recent finding that prohibitin associates with the mIgM antigen receptor in B-lymphocytes as judged by coprecipitation of prohibitin with mIgM in cell lysates [18] is difficult to interpret in the light of our results. More data are clearly necessary to accomodate these findings with the function of prohibitin.

The intriguing question raised by our findings is how the mitochondrial localization of prohibitin is related to its putative tumor suppressor activity. Whatever the mechanism of action of prohibitin, ultimately, to act as an antioncogene, it should lead to transduction of an inhibitory signal for cell division. The strongest evidence for a link between the function of the prohibitin gene product and development and/or progression of neoplasia comes from a case of sporadic breast carcinoma where it was obvious that in the tumor cells neither of the alleles could produce a normal prohibitin protein [7]. Provided that this biallelic loss of function plays a role in the pathogenesis of breast cancer prohibitin would be the first mitochondrially localized protein acting as a tumor suppressor.

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