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A cell death assay for assessing the mitochondrial targeting of proteins

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Abstract

The mitochondrial proteome comprises 1,000 to 1,500 proteins, in addition to proteins for which the mitochondrial localization is uncertain. About 800 diseases have been linked with mutations in mitochondrial proteins. We devised a cell survival assay for assessing the mitochondrial localization in a high-throughput format. This protocol allows us to assess the mitochondrial localization of proteins and their mutants, and to identify drugs and nutrients that modulate the mitochondrial targeting of proteins. The assay works equally well for proteins directed to the outer mitochondrial membrane, inner mitochondrial membrane mitochondrial and mitochondrial matrix, as demonstrated by assessing the mitochondrial targeting of the following proteins: carnitine palmitoyl transferase 1 (consensus sequence and R123C mutant), acetyl-CoA carboxylase 2, uncoupling protein 1, and holocarboxylase synthetase. Our screen may be useful for linking the mitochondrial proteome with rare diseases, and for devising drug and nutrition based strategies for altering the mitochondrial targeting of proteins.

Keywords

mitochondria; proteome; high throughput screen

Introductory Statement

The mitochondrial proteome comprises 1,000 to 1,500 proteins [1] (<http://locate.imb.uq.edu.au>), not counting several hundred proteins for which the mitochondrial localization is uncertain (<http://www.mitoproteome.org>). Mitochondrial DNA is coding for

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only 13 proteins, i.e., the vast majority of mitochondrial proteins are encoded by nuclear DNA [1]. The biological importance of mitochondrial proteins is undisputed and includes processes such as apoptosis, respiratory chain, and β -oxidation of fatty acids (<http://www.mitoproteome.org>) [1, 2]. Mitochondrial proteins have been linked with about 800 diseases including diabetes mellitus, encephalopathies, stroke, and Alzheimer's and Parkinson's disease (<http://bioinfo.nist.gov/hmpd/index.html>). Despite the quantitative and qualitative importance of mitochondrial proteins in cell biology and the genesis of diseases there are still a large number of open questions that need to be addressed, as highlighted in the following examples. Possible impacts, both adverse and beneficial, of altering the mitochondrial targeting of proteins through xenobiotic and nutritional factors are poorly understood. The tissue specificity of the mitochondrial proteome is largely unknown and, along the same lines, the mitochondrial localization and function in mitochondrial biology are unknown for a meaningful number of proteins. Likewise, the extent to which mutations affect mitochondrial localization is unknown for the vast majority of proteins. The identification of mitochondrial proteins is further complicated by the low abundance of many mitochondrial proteins, the potential impact of developmental stages, and because no single targeting sequence directs the mitochondrial import of proteins [1, 2].

We have developed a cell death-based assay in a high-throughput format to position investigators for answering many of the questions raised above, and tested the assay with a number of proteins to demonstrate feasibility. Briefly, the protocol is based on transfecting human cells with plasmids coding for putative mitochondrial proteins fused to the cell death peptide KLAKLAKKLAKLAK [3]. The peptide KLAKLAKKLAKLAK disrupts the mitochondrial membrane potential and causes cell death when brought in close physical proximity to the mitochondrial membrane through the fused protein. Cell survival is assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay [4].

The following proteins were included in our analysis in order to have a broad representation of proteins of uncertain mitochondrial localization, proteins localizing in distinct mitochondrial compartments, proteins with key roles in fatty acid metabolism, protein mutants, and proteins implicated in rare diseases. (a) The protein biotin ligase holocarboxylase synthetase (HLCS) is a protein of uncertain mitochondrial localization. HLCS is not listed in databases of mitochondrial proteins (<http://locate.imb.uq.edu.au> <http://www.mitoproteome.org>) [5], yet it catalyzes the covalent binding of the coenzyme biotin to the enzymes pyruvate carboxylase (PC), 3-methylcrotonyl-CoA carboxylase, and propionyl-CoA carboxylase [6], which reside in the mitochondrial matrix. Western blots of mitochondrial HLCS are inconclusive [7]. Mutations on HLCS gene may lead to a condition determined as multiple carboxylase deficiency (MCD) [8]. MCD patients generally exhibit low activities of all five biotin-dependent carboxylases and the incidence of HLCS deficiency is less than 1 of 100 000 live births in Japan [9]. (b) Carnitine palmitoyltransferase 1 (CPT1) and acetyl-CoA carboxylase 2 (ACC2) localize to the outer mitochondrial membrane and play essential roles as positive and negative regulators, respectively, of the mitochondrial import of fatty acids [10, 11]; due to their role in the regulation of fatty acid metabolism they are targets for the treatment of the metabolic syndrome [12]. The C367T (R123C) mutation in CPT1 (CPT1_{R123Cmut}) maps near a

mitochondrial transmembrane sequence, appears to affect the mitochondrial processing of CPT1, and causes a 30% loss in CPT1 activity [13]. (c) Uncoupling protein 1 (UCP1) localizes to the inner mitochondrial membrane in brown adipose tissue, where it uncouples oxidative phosphorylation from adenosine triphosphate synthesis with energy being dissipated as heat [14].

Materials and Methods

Materials

Reagents for creating plasmids for stable cell lines and the T-Rex™-293 cell line were purchased from Invitrogen (T-Rex™-293 System; Carlsbad, CA). Tetracycline-free fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA). Doxycycline hyclate and resveratrol compounds were purchased from Sigma (St. Louis, MO). Hygromycin B and blasticidin S HCl were obtained from Invitrogen (Carlsbad, CA). Hamaelis crude extracts were obtained from Dr. Frank Döring, University of Kiel [15].

Plasmids

A clone coding for full-length human ACC2 was obtained from the Arizona State University Biodesign Institute plasmid repository (clone HsCD00353997) and subcloned into the pcDNA-DEST47 mammalian expression vector using Gateway® Recombination cloning technology (Invitrogen), thereby creating ACC2 tagged with green fluorescence protein (GFP) in the C-terminus. This plasmid served as template for creating the various plasmids built around ACC2. First, a sequence coding for the 416 N-terminal amino acids in ACC2 (GenBank Accession Number NM_001093.3), known to be sufficient for mediating mitochondrial targeting [11], was PCR amplified using primers 5'-GGGACTCGAGATGGTCTTGCTTCTTTGTCTATCTTGTCTGATT-3' (forward) and 5'-ATTTGAATTCGCTGCAGATCATCTTCTGTCCACTCCAC-3' (reverse). The PCR product was inserted into the pAcGFP1-N1 vector (Clontech Laboratories, Mount View, CA) using *XhoI* and *EcoRI*, thereby creating plasmid ACC2 (Fig. 1b). For creating plasmid ACC2-KLAK, the following oligonucleotides were annealed: 5'-AGATGGGAGGAAAGCTGGCCAAGCTGGCCAAGAAGCTGGCCAAGCTGGCCAAGG-3' (forward) and 5'-CCTTGGCCAGCTTGGCCAGCTTCTTGGCCAGCTTGGCCAGCTTTCCTCCCATCT-3' (reverse); they code for the cell death peptide KLAKLAKKLAKLAK and two N-terminal linker glycines, which are important to minimize steric hindrance [3]. The sequence was inserted downstream from the ACC2 reading frame in plasmid ACC2 using *SmaI* and blunt end cloning (ACC2-KLAK, Fig. 1a). Plasmid ACC2-DLSL was created using the same procedure as described for ACC2-KLAK, but using oligonucleotides coding for the control peptide DLSLARLATARLAI (DLSL): 5' are important to minimize steric hindrance [3]. The sequence was inserted downstream from the ACC2 reading frame in plasmid ACC2 using *SmaI* and blunt end cloning (ACC2-KLAK, Fig. 1a). Plasmid ACC2-DLSL was created using the same procedure as described for -AGATGGGAGGAGACCTGAGCCTGGCCAGGCTGGCCACCGCCAGGCTGGCCATCG-3' (forward) and 5'-CGATGGCCAGCCTGGCGGTGGCCAGCCTGGCCAGGCTCAGGTCTCCTCCCATCT-3

' (reverse). The control plasmids KLAKE and DLSL were created by inserting the oligonucleotides coding for (GG)KLAKEKLAKEKLAKE and (GG)DLSLARLARLAR into the *SmaI* site in the pAcGFP1-N1 vector.

Other plasmids were built on the backbone of plasmid KLAKE (Fig. 1a). Briefly, full-length human HLCS (GenBank Accession Number NM 000411.6) was amplified using the following oligonucleotides and the PCR product was inserted into plasmid KLAKE using *XhoI* and *EcoRI*: 5'-GCACCTCGAGATGGAAGATAGACTCCACATGG-3' (forward) and 5'-CGTAGAATTCGCCGCCGTTTGGGGAGGAT-3' (reverse), thereby creating plasmid HLCS-KLAKE. Full-length human CPT1 (GenBank Accession Number NM 001876.3) was amplified using the following oligonucleotides and the PCR product was inserted into plasmid KLAKE using *XhoI* and *EcoRI*: 5'-

ACTGCTCGAGATGGCAGAAGCTCACCAAGCTGTGGCCTTTCAGTTC-3' (forward) and 5'-

ACTGGAATTCGCTTTTTTGAATTAGAACTGAGACCAAACAAAGTGATGATGTCAGTCAT-3' (reverse), thereby creating plasmid CPT1-KLAKE. Full-length human UCP (GenBank Accession Number BC098352.1) was amplified using the following oligonucleotides and the PCR product was inserted into plasmid KLAKE using *XhoI* and *EcoRI*: 5'-ACTGCTCGAGATGGGGGGCCTGACAGCCTCGGAC-3' (forward) and 5'-ACTGGAATTCGTGTGGCACAGTCCATAGTCTGCCTTGACTTTGACAGTTCTCGTTT-3' (reverse), thereby creating plasmid UCP1-KLAKE. Plasmid CPT1_{R123Cmut}-KLAKE was created by site-directed mutagenesis of plasmid CPT1-KLAKE using the Geneart™ site-directed mutagenesis system kit (Invitrogen, Carlsbad, CA) and primers 5'-

CCTCATCGTCACCATGTGCTACTCCCTGAAAGT-3' (forward) and 5'-ACTTTTCAGGGAGTAGCACATGGTGACGATGAGG-3' (reverse). pcDNA5-FRT/TO-ACC2-GFP-KLAKE_{Tet-On} and pcDNA5-FRT/TO-GFP-KLAKE_{Tet-On} were constructed by excising the ACC2-GFP-KLAKE_{Tet-On} or ACC2-GFP-KLAKE_{Tet-On} fragment from the pAcGFP backbone using the *BglII* and *NotI* restriction sites and cloning into the pcDNA5-FRT/TO at the *BamHI/NotI* sites. pcDNA5-FRT/TO-GFP_{Tet-On} construction was previously described [16]. The identities of inserts in each plasmid were confirmed by sequencing.

Cell death plasmids

Human cells were transfected with a plasmid coding for the 416 N-terminal amino acids in ACC2 fused to the peptide KLAKEKLAKEKLAKE through two linker glycines, thereby creating plasmid ACC2-KLAKE (Fig. 1). The design of this plasmid is based on a three-part rationale. First, a construct based on the 416 N-terminal amino acids has been used successfully to demonstrate mitochondrial ACC2 anchoring [11]. Second, previous studies suggest that the peptide KLAKEKLAKEKLAKE disrupts the mitochondrial membrane potential and causes cell death when brought in close physical proximity to the mitochondrial membrane [3]. Third, the use of linker glycines minimizes steric hindrance [3]. In this study we investigated compounds that mask the mitochondrial targeting signal in the ACC2 N-terminus impart cell survival through preventing physical contact between KLAKEKLAKEKLAKE and mitochondria. We assessed the cell survival using the MTT assay [4].

Theoretically, transient overexpression of proteins might cause artifacts in protein localization. Plasmid ACC2-KLAK codes for the N-terminus of ACC2 fused to the death signal peptide KLAKLAKKLAKLAK. Plasmid KLAK codes for peptide KLAKLAKKLAKLAK, and plasmid ACC2 codes for the N-terminus of ACC2. KLAK serves as a negative control, because GFP localizes to the cell cytoplasm [17, 18]. Additional controls were prepared based on the rationale that the ability of the KLAKLAKKLAKLAK peptide to cause cell death depends on the formation of an α -helix. Negative controls were prepared by fusing the N-terminus of ACC2 to a sequence coding for peptide DLSLARLATARLAI, which does not form an α -helix or cause cell death [3]. Plasmid ACC2-DLSL codes for the N-terminus of ACC2 fused to the control peptide DLSLARLATARLAI, and plasmid DLSL codes for peptide DLSLARLATARLAI fused to GFP. Importantly, all vectors carry the neoR resistance gene to allow selection of stably transformed cells, and also code for a GFP tag to allow for assessing expression and intracellular trafficking by confocal microscopy. “Gly-Gly” denotes a glycine dipeptide, inserted to minimize steric hindrance [3]. Finally, a clone coding for full-length human ACC2 was obtained from the Arizona State University Biodesign Institute plasmid repository (clone HsCD00353997) and subcloned into the pcDNA-DEST47 mammalian expression vector using Gateway® Recombination cloning technology (Invitrogen), thereby creating ACC2 tagged with GFP in the C-terminus (plasmid Full-length ACC2).

Production of Stable Cell Lines

Stable integration of GFP, GFP-KLAK, and GFP-KLAK-ACC2 in HEK293 human embryonic kidney cells was accomplished using the Flp-In System, which employs 293 T-Rex cells containing two stably integrated plasmids. The first plasmid pcDNA6/TR constitutively expresses TetR; a single integrated copy of the second plasmid pFRT/lacZeo contains the yeast FRT recombination site. These cells were co-transfected with pcDNA5-FRT/TO-ACC2-GFP-KLAKTet-On, pcDNA5-FRT/TO- GFP-KLAKTet-On, or pcDNA5-FRT/TO-GFPTet-On and pOG44, a plasmid expressing the *Saccharomyces cerevisiae* Flp recombinase. Expression of Flp mediates a single recombination event between the FRT site in pcDNA5/FRT/TO plasmids and the FRT site integrated into the HEK293 genome, thus ensuring that the resulting cells express each of their exogenous genes from a single copy at the same genetic locus. Properly targeted cells were selected by growth in 200 μ g/ml hygromycin and 7.5 μ g/ml blasticidin. Stably transformed HEK293 cells were grown in medium containing tetracycline-free fetal bovine serum; genes were activated by doxycycline (5 μ g/mL of media) when applicable. Cell viability was assayed by trypan blue exclusion and visualized in hemocytometer.

Cell cultures

HEK293 cells were cultured in Dubelcco's Modified Eagle Medium (DMEM) following the vendor's instructions as described previously [19]. Where indicated, HEK293 cells were cultured in biotin-defined DMEM containing 0.025 nM, 0.25 nM, and 10 nM biotin, representing plasma concentrations observed in biotin-deficient, biotin-sufficient, and biotin-supplemented persons; biotin-defined media were prepared as described previously [19]. Previous studies suggest that incubating mammalian cells in biotin-defined media for 48 h is sufficient to establish new steady state concentrations of biotin [20]. HEK293 cells were first

cultured in biotin-sufficient media (0.25 nM biotin) for three days, followed by culture in biotin-defined media for 7 days assessing the effects of biotin on the mitochondrial localization of the biotin protein ligase, HLCS. Efficacy of biotin treatment was assessed by probing the abundance of biotinylated carboxylases using gel electrophoresis and streptavidin blots [20, 21]. As loading control total PC were probed using rabbit anti-human PC.

Assay principle and model proteins

Human cells were transfected with a plasmid coding for mitochondrial proteins fused to the peptide KLAKLAKKLAKLAK through two linker glycines, thereby creating plasmids HLCS-KLAK, CPT1-KLAK, CPT1_{R123Cmut}-KLAK, ACC2-KLAK and UCP1-KLAK (Fig. 1a). Plasmids also code for a GFP tag to allow monitoring for transfection efficiency. The peptide KLAKLAKKLAKLAK disrupts the mitochondrial membrane potential and causes cell death when brought in close physical proximity to the mitochondrial membrane (Supplementary Fig. 1) [3]. Plasmid KLAK codes for peptide KLAKLAKKLAKLAK with no fusion protein (negative control; Fig. 1a). Cells were transfected with these plasmids and cell survival was assessed using the MTT assay [4]. Xenobiotics and nutrients that increase or decrease the targeting of a protein to mitochondria will cause a change in the physical proximity of KLAKLAKKLAKLAK to the mitochondrial membrane, thereby decreasing or increasing, respectively, cell survival.

ACC2 was used as the primary model for assay optimization and demonstrating feasibility. ACC2 is a 280-kDa protein which, based on the plasmid's size, results in low transfection efficiency [11]. Previous studies suggest that a construct based on the 416 N-terminal amino acids in ACC2 is sufficient to mediate anchoring of ACC2 in the outer mitochondrial membrane [11]. Therefore, studies of ACC2 were conducted using the protein's N-terminus. In studies of ACC2 we included the following controls, in addition to KLAK (Fig. 1b): (a) plasmid ACC2, coding for the N-terminus in ACC2; (b) plasmid DLSL, coding for peptide DLSLARLATARLAI; note that the ability of the KLAKLAKKLAKLAK peptide to cause cell death depends on the formation of an α -helix and that DLSLARLATARLAI does not form an α -helix [3]; and (c) plasmid ACC2-DLSL coding for the N-terminus of ACC2 fused to the control peptide DLSLARLATARLAI.

Cell survival assay

Unless noted otherwise, cells were seeded in 96-well plates (50,000 cells/well) and allowed to adhere for 24 h. Cells were transfected with the indicated plasmid and treated, if applicable, with compounds that prevent mitochondrial targeting of proteins. After 24 h, cell survival (respiratory activity) was assessed spectrophotometrically using the MTT assay at 562 nm and an incubation time of 1 h [4]. Where applicable, values are reported as MTT, i.e., the absorbance of cells transfected with plasmid KLAK minus the absorbance of cells transfected with a plasmid in which KLAK is fused to a protein.

Confocal microscopy

HEK293 cells were grown to about 65% confluence in the microscopy core facility at the University of Nebraska-Lincoln. Cells were transfected with ACC2 (Fig. 1b) and treated

with an aqueous extract of 3-chlorogenic acid (10 μ M final concentration) or solvent for 3 days. Mitochondria were stained using Mito Tracker® Red CMXRos. Images were collected at 579/599 nm (mitochondria) and 475/509 nm for GFP-tagged ACC2.

Quantitative real-time PCR

mRNA was purified from HEK293 cells using RNA MicroPrep™ spin columns and reverse transcribed following manufacturer's instruction (Zymo Research, Irvine, CA). GFP cDNA was amplified using forward primer 5'-GATAACCACTACCTGTCCAC-3' and reverse primer 5'-CGAAGCCGAAGTAGATCATGTG-3' and quantified using the C_t method [22] and normalizing for amplification efficiency using glyceraldehyde-3-phosphate dehydrogenase [23].

Statistics

One-way ANOVA and the Fisher' PLSD test for posthoc comparisons were used when more than two treatments were compared. GraphPad Prism 6.0 was used for calculations. Data are reported as means $\pm$ SD. Differences were considered statistically significant if $p < 0.05$.

Results

Implementation of the ACC2-KLAK assay

In a first proof-of-concept experiment, HEK293 cells were seeded in 6-well plates and transiently transfected with the cell death plasmid ACC2-KLAK and control plasmids. Cell viability was assessed by phase contrast microscopy and a green filter 24 h after transfection. The vast majority of cells that had been transfected with ACC2-KLAK had died, whereas cells treated with transfection reagent and negative control plasmids KLAK, ACC2, ACC2-DLSL, and DLSL continued to proliferate (Fig. 2 and 3). Subsequent assays were conducted in 96-well plates for a total time of about 24 h (Supplementary Note 1), and plasmid KLAK was used as the primary negative control.

After identifying the linear range for incubation time and cell number in the MTT assay (Supplementary Fig. 2), HEK293 cells were transiently transfected with ACC2-KLAK or KLAK, and MTT assays were conducted at timed intervals after transfection. The difference in the absorbance in cells transfected with ACC2-KLAK and KLAK (MTT) was maximal 24 h after transfection (Fig. 4). We assessed the quality and precision of the assay by using Z-Prime and Coefficient of Variation (CV%), respectively, in 96-well plates using the same conditions as described in the legend to Fig. 4 [24]; these are important variables when assessing the feasibility of an assay for use in a high-throughput format (Supplementary Note 2). Z-Prime was 0.77 and CV% was 4.8%.

Application to identifying mitochondrial proteins and its regulation by small molecules

Next, the assay was applied to assessing the mitochondrial localization of human proteins in HEK293 cells. Rapid cell death was evident when cells were transfected with KLAKLAKKLAKLAK fusion constructs of CPT1, ACC2, UCP1, and HLCS, judged by MTT assay (Table 1). Mitochondrial targeting was not impaired for the CPT1_{R123C} mutant, indicating that the activity loss is not related to mitochondrial localization. Transfection

efficiency was not significantly different among the various constructs, judged by the expression of GFP mRNA (Supplementary Table 1).

KLAKLAKKLAKLAK fusion constructs are useful for detecting effects of nutrients and drugs on the mitochondrial targeting of proteins, as illustrated by the following examples. (a) We determined whether biotin availability affects the mitochondrial localization of the protein biotin ligase HLCS. The mitochondrial localization of HLCS increased moderately by about 10% in biotin-supplemented HEK293 cells compared with biotin-sufficient cells, evidenced by a decrease in cell growth judged by MTT assay (abs. at 562 nm): 0.93 ± 0.07 vs. 1.03 ± 0.06 (Supplementary Fig. 3). Efficacy of biotin depletion and supplementation was confirmed by assessing the abundance of biotinylated holocarboxylases, which is a well-established marker for biotin nutrition in cell cultures and humans (Supplementary Fig. 4) [20, 21].

(b) Preliminary studies suggest that extracts from *Hamamelis virginiana* (witch hazel) inhibits ACC2-KLAK induced HEK293 cell death, presumably by disrupting the anchoring of ACC2 in the outer mitochondrial membrane (Supplementary Fig. 5); we determined which of the classes of polyphenols in the extract caused this effect. The effects of *Hamamelis* compounds on the death of HEK293 cells transfected with ACC2-KLAK was further investigated using chemically defined compounds from *Hamamelis*. Leaf extracts from *Hamamelis virginiana* are a rich source of galloyl hexoses (~1,000 mg/100 g fresh leaves, predominantly octa- and nongalloyl hexoses), flavonols (~90 mg/100g, predominantly kaempferol hexosides I and II and quercetin hexoside), and chlorogenic acids (~60 mg/100 g, predominantly 3-caffeoylquinic acid and 5-caffeoylquinic acid) [25]. When HEK293 cells were transfected with ACC2-KLAK and cultured with chemically pure compounds representing the three major polyphenol classes in *Hamamelis*, 3-chlorogenic acid caused a 54% increase in cell survival compared with the vehicle control (Fig. 5a); in a similar way, quercetin-3-rutinoside (rutin) and gallic acid caused increases (38 and 28%, respectively) in HEK293 cell survival. Despite all compounds offering significant increase in cell survival, we chose 3-chlorogenic acid for further analysis due to numerically higher potential. The survival-enhancing effects were dose dependent for 3-chlorogenic acid (Fig. 5b). Theoretically, it is possible that these compounds activate cell survival pathways rather than inhibit the targeting of ACC2-KLAKKLAKLAK to the mitochondrial membrane. To determine whether the observed effects were caused by altered mitochondrial binding of ACC2, HEK293 cells were transfected with plasmid ACC2 (Fig. 1b) and mitochondrial targeting was assessed by tracking the GFP tag by confocal microscopy. When cells were treated with an aqueous extract of 3-chlorogenic acid (10 μ M, final concentration), the GFP signal associated with mitochondria decreased compared with vehicle controls, evidenced by the loss of yellow signal in chlorogenic acid-treated cells compared with controls in the merged images (Fig. 5c,d).

Tet-On strategy for assessing the effects of long-term treatment on mitochondrial localization

Under certain circumstances it may be desirable to assess the effects of continued exposure to drugs and nutrients on the mitochondrial localization of proteins. Such studies are not

feasible when using an approach that involves plasmids with constitutively active promoters such as those described above, because KLAKLAKKLAKLAK fusion proteins kill cells within about 24 h (Fig. 2 and 3); the above assays are intended for use in high-throughput screens that do not require lengthy treatment of cells. We determined whether Tet-On protocols offer a viable strategy for suppressing ACC2-KLAK while treating cells with reagents of choice for extended periods of time. GFP-tagged ACC2-KLAK was subcloned into a tetracycline-inducible plasmid (pcDNA5-FRT/TO-ACC2,GFP-KLAK_{Tet-On}) and stably integrated into the FRT locus in the HEK293-Flp/TRex cell genome (Invitrogen); these cells also express the tetracycline repressor. In the absence of tetracycline, ACC2,GFP-KLAK_{Tet-On} cells were viable during the entire observation period of 15 days, but the cumulative cell numbers were about one order of magnitude lower than those of GFP_{Tet-On} and KLAK_{Tet-On} controls (Supplementary Fig. 6a). The ACC2,GFP-KLAK_{Tet-On} cells persisted proliferating for 3 days after adding doxycycline to the medium, as the same occurred for KLAK_{Tet-On} (Supplementary Fig. 6b). The slow growth of ACC2,GFP-KLAK_{Tet-On} cells in the absence of doxycycline was probably caused by leaky expression of ACC2-KLAK-GFP, evidenced by a distinct GFP signal in ACC2,GFP-KLAK_{Tet-On} cells (Supplementary Fig. 6b).

Discussion

We have developed a new protocol for the identification of mitochondrial proteins and the assessment of xenobiotic and nutritional factors and mutations that alter the mitochondrial localization of proteins. The protocol was validated by testing five known and putative mitochondrial proteins and identifies proteins regardless of the mitochondrial compartment they are associated with. Our cell death assay has considerable strengths when compared with traditional protocols for mitochondrial proteins such as immunocytochemistry and western blot analysis. First, the assay is suited for high-throughput screens. The entire screen can be carried out in one single plate, using 96-well or 384-well plate formats, without requiring the transfer of samples to other plates or washing plates between steps. There is no need for using costly reagents such as antibodies or specialized equipment which renders the assay an economical approach to studies of the mitochondrial proteome and its regulation. Second, the assay yields semi-quantitative results because it is coupled to the MTT assay or similar assays. The assay does not permit quantifying the amount of protein associated with the mitochondrial membrane. In contrast, most immunocytochemistry are designed to yield qualitative data. Third, the KLAKLAKKLAKLAK-based assay is conducted in live cells which allows for conducting time-response studies of drugs that affect the mitochondrial localization of proteins and prevents assay artifacts caused by protein degradation during purification for western blots. Note that similarly powerful protocols have been published by Ting and co-workers and present a viable alternative to the KLAK-based protocol [26, 27].

We envision numerous meaningful applications for this assay. Examples include the (a) identification of food-borne or synthetic molecules that mask the mitochondrial anchoring motif in the ACC2 N-terminus [11], thereby increasing the mitochondrial uptake of fatty acids for subsequent β -oxidation and causing a lean phenotype [11, 28, 29]; (b) identification of the entire mitochondrial proteome in distinct tissues or cell lines; (c) identification of mutations and polymorphisms that alter the mitochondrial localization of

proteins; and (d) drug screens for inhibiting the localization of γ -secretase subunits (presenilins 1 and 2, nicastrin, APH-1, and PEN-2) in the *mitochondria-associated membranes* space to combat Alzheimer's disease [30-32].

The KLA-based analysis of mitochondrial proteins is not a viable choice for identifying the localization of proteins in distinct mitochondrial compartments. For example, the assay produced an equally strong signal for proteins that reside in the inner (UCP1) and outer (ACC2, CPT1) membrane. This being said, the assay is a mere screening tool for the overall mitochondrial targeting of proteins, identification of mitochondrial localization signals, and discovery of compounds that inhibit mitochondrial targeting of proteins.

There also is the possibility that overexpression of proteins might cause aberrant localization of proteins. We titrated the amount of HLCS protein in an attempt to provide a point reference for users of mitochondrial targeting assays. Note that cells stably transfected with ACC2-KLA behave similar to transiently transduced cells, and that the level of expression typically is considerably small in stable transformations compared with transient transformations.

In its current form, the assay does not provide a firm cut-off, *i.e.*, an absolute or percent change in absorbance in the MTT assay, when screening for compounds that alter mitochondrial localization of ACC2. These cut-offs need to be defined by users, depending on the experimental conditions and objectives of their studies. Investigators are cautioned that some compounds might affect cell proliferation and death and are advised to assess any such effects in the absence of KLA plasmids.

In retrospect, we would advise users of this assay to use a mitochondrial stain other than CMXRos mitotracker, because of its dependence on mitochondrial integrity. We would expect dyes that do not depend on membrane potential produce a much stronger counterstain than what we achieved by using CMXRos.

Note that Tet-On protocols are of limited use when seeking to culturing cells stably transformed with KLA_{KLAKKLAK} fusion proteins. The cumulative number of cells of ACC2,GFP-KLA_{Tet-On} cells was about one order of magnitude smaller than control cells over a 15-day period. The low productivity of ACC2,GFP-KLA_{Tet-On} cell cultures is due to a leaky expression of the Tet-On system used in this study and the strong death signal of KLA_{KLAKKLAK}. This observation is consistent with the leaky expression of Tet-On system reported in previous studies [33, 34]. The application of KLA_{KLAKKLAK}-based fusion constructs in the spatio-temporal analysis of mitochondrial proteins will have to await the creation of protocols that prevent transgene expression in solvent controls.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
HLCS	holocarboxylase synthetase
MCD	multiple carboxylase deficiency
CPT1	carnitine palmitoyltransferase 1
ACC2	acetyl-CoA carboxylase 2

PC	pyruvate carboxylase
CPT1_{R123Cmut}	C367T mutation in CPT1
UCP1	uncoupling protein 1
GFP	green fluorescence protein
KLAK	KLAKLAKKLAKLAK peptide
DLSL	DLSLARLATARLAI peptide
DMEM	Dulbecco's Modified Eagle Medium
CV%	Coefficient of Variation

Figure 1.
Schematic of plasmids tested in the cell death assay. **(a)** Protein-KLAKLAKKLAKLAK fusion proteins. **(b)** Negative controls.

Figure 2.

Transient transfection with plasmid ACC2-KLAK causes HEK293 cell death. Cells were seeded in 6-well plates (10^6 cells per well) and transfected with ACC2-KLAK (a), solvent control (b), KLAK (c), ACC2 (d), ACC2-DLSL (e), and DLSL (f). Cells were visualized using phase contrast microscopy and a green filter immediately after transfection.

Figure 3.

Transient transfection with plasmid ACC2-KLAK causes HEK293 cell death. Cells were seeded in 6-well plates (10^6 cells per well) and transfected with ACC2-KLAK (a), solvent control (b), KLAK (c), ACC2 (d), ACC2-DLSL (e), and DLSL (f). Cells were visualized using phase contrast microscopy and a green filter 24 h after transfection.

Figure 4.

Time course of MTT in ACC2-KLAK assays. MTT = absorbance of HEK293 cells transfected with KLAK minus the absorbance in cells transfected with ACC2-KLAK. 50,000 cells were seeded per well in a 96-well plate, and transfections were conducted after letting the cells adhere overnight; at timed intervals, cells were incubated with MTT for 1 h. ^{a,b}Bars not sharing the same superscript are significantly different; $p < 0.05$. Values are means $\pm$ SD. N=3 independent repeats in independent samples

Figure 5.

3-Chlorogenic acid increases the survival of HEK293 cells transfected with ACC2-KLAK.

(a) Parent compounds of the major polyphenol classes in *Hamamelis virginiana* were tested at equimolar concentrations (10 μM) and compared with vehicle controls KLAK and ACC2-KLAK exclusive transfection. (b) Dose-dependent effects of 3-chlorogenic acid. N=3 independent samples. N=3 independent repeats in independent samples. ^{a,b,c,d} Bars not sharing the same superscript are significantly different; $p < 0.05$. Values are means $\pm$ SD.

Values are means $\pm$ SD. N=3 independent repeats in independent samples. (c,d) Mitochondrial localization of GFP-tagged ACC2 N-terminus in HEK293 cells treated with 10 μM 3-chlorogenic acid (c) and vehicle controls (d). Confocal microscopy was used to track GFP with laser at 488nm (panel 1) and mitochondria (Mito Tracker® Red CMXRos with laser at 543 nm, panel 2); merged and phase contrast images are shown in panels 3 and 4, respectively. The lens used to obtain the images was UPlanSApo 60 $\times$ /1.35 in oil immersion.

Table 1
Cell death caused by fusion proteins of mitochondrial proteins and the
KLAKLAKKLAKLAK peptide

Plasmid	MTT abs.
KLAK	0.932±0.065 ^a
CPT1-KLAK	0.721±0.056 ^b
ACC2-KLAK	0.563±0.034 ^b
UCP1-KLAK	0.647±0.072 ^b
HLCS-KLAK	0.571±0.089 ^b
CPT1 _{R123Cmut} -KLAK	0.668±0.053 ^b

^{a,b} Entries not sharing the same superscript are significantly different (p<0.0001). N=6 independent repeats in independent samples.