ABSTRACT

Mutations in subunits of succinyl-CoA synthetase/ligase (SCS), a component of the citric acid cycle, are associated with mitochondrial encephalomyopathy, elevation of methylmalonic acid (MMA), and mitochondrial DNA (mtDNA) depletion. A FACS-based retroviral-mediated gene trap mutagenesis screen in mouse embryonic stem (ES) cells for abnormal mitochondrial phenotypes identified a gene trap allele of Sucla2 (Sucla2SA), which was used to generate transgenic mice. Sucla2 encodes the ADP-specific β-subunit isofrom of SCS. Sucla2SA homozygotes exhibited recessive lethality, with most mutants dying late in gestation (e18.5). Mutant placenta and embryonic (e17.5) brain, heart and muscle showed varying degrees of mtDNA depletion (20-60%). However, there was no mtDNA depletion in mutant liver, where the gene is not normally expressed. Elevated levels of mRNA were observed in embryonic brain. SCS-deficient mouse embryonic fibroblasts (MEFs) demonstrated a 50% reduction in mtDNA content compared with wild-type MEFs. The mtDNA depletion resulted in reduced steady state levels of mtDNA encoded proteins and multiple respiratory chain deficiencies. mtDNA content could be restored by reintroduction of Sucla2. This mouse model of SCS deficiency and mtDNA depletion promises to provide insights into the pathogenesis of mitochondrial diseases with mtDNA depletion and into the biology of mtDNA maintenance. In addition, this report demonstrates the power of a genetic screen that combines gene trap mutagenesis and FACS analysis in mouse ES cells to identify mitochondrial phenotypes and to develop animal models of mitochondrial dysfunction.

KEY WORDS: TCA cycle, Mitochondrial DNA depletion, Gene trap, Mitochondria

INTRODUCTION

Mitochondrial disease is a significant cause of heritable multiorgan dysfunction. Current epidemiological evidence suggests that the prevalence of mitochondrial disorders might be as high as 1 in 5000, making mitochondrial disease one of the more common genetic causes of encephalomyopathies and multisystem disease (Schaefer et al., 2004; Elliott et al., 2008; Schaefer et al., 2008). Despite important insights into clinical, biochemical and molecular features of these disorders, the underlying molecular pathogenesis remains poorly understood and no clearly effective therapies exist. Mitochondria contain their own genome that consists of a multicopy, ~16.4-kilobase circular chromosome. This mitochondrial DNA (mtDNA) encodes 13 polypeptides that are subunits of various respiratory chain complexes as well as 22 tRNAs and two rRNAs required for mitochondrial protein translation. The mitochondrial proteome consists of ~1300 proteins, therefore the remaining 99% of mitochondrial proteins are nuclear encoded, including all of the protein machinery required for mtDNA replication, maintenance, transcription and translation (Calvo and Mootha, 2010). Mitochondrial disease can be caused by mutations in mtDNA or in nuclear-encoded genes, with the majority of pediatric cases of mitochondrial disease presumably caused by recessive mutations in nuclear-encoded genes, of which only a small fraction are identified (Haas et al., 2008).

Mitochondrial encephalomyopathy with mtDNA depletion represents an important subset of mitochondrial diseases and is defined by a global or tissue-specific reduction in mtDNA copy number. Over the last decade, mitochondrial diseases associated with mtDNA depletion have been described and a number of causative genes identified (Graham, 2012). These clinically heterogeneous disorders are autosomal recessive and encompass a wide spectrum of clinical features, including combinations of infantile or childhood encephalopathy with severe intellectual disability, myopathy, cardiomyopathy and hepatopathy. Nuclear-encoded genes associated with mtDNA depletion syndromes include genes important for mtDNA replication (POLG, TWINKLE), regulation of mitochondrial nucleotide pools (DGUOK, TP, TK2, RRMB2B) and genes with poorly defined functions related to mtDNA maintenance, including, MPV17 and, interestingly, subunits of the Kreb’s cycle enzyme succinyl-CoA synthetase, SCS (SUCLG1, SUCLG2) (Suomalainen and Isohanni, 2010). Although animal models have been reported for many of these genes (Haraguchi et al., 2002; Kimura et al., 2003; Hance et al., 2005; Tyynismaa et al., 2002; Akman et al., 2008; Martinez-Azorin et al., 2008; López et al., 2009; Viscomi et al., 2009), there is currently no reported animal model for SCS-dependent mtDNA depletion.

SCS is the TCA cycle enzyme responsible for the conversion of succinyl-CoA to succinate in the mitochondrial matrix and is coupled to the phosphorylation of GDP or ADP, thereby providing the only ‘substrate level’ phosphorylation in the TCA cycle. SCS is a heterodimer, composed of a catalytic α-subunit (SUCLG1) and a dNDP-binding β-subunit. There are two isoforms of the β-subunit:
Mitochondrial disease associated with loss of cellular mitochondrial DNA content (mtDNA depletion) is characterized by a global or tissue-specific reduction in mtDNA copy number. Mitochondrial disease with mtDNA depletion can be caused by mutations in one of several genes and can cause dysfunction of one or more organs, including brain, heart, skeletal muscle and liver. SUCLA2 is one of these genes and encodes the ADP-specific β-subunit of succinyl-CoA synthetase (SCS), an enzyme responsible for conversion of succinyl-CoA to succinate in the Krebs (citric acid) cycle. Patients with SUCLA2 mutations generally exhibit intellectual disability, severe low muscle tone, dystonia and deafness. Mild elevation of methylmalonic acid (MMA) and loss of mtDNA in muscle are considered hallmarks of SUCLA2 deficiency. Currently, animal models for SUCLA2 deficiency are lacking, the underlying disease mechanisms are poorly understood and no efficacious treatments are available.

Results

By performing a FACS-based retroviral-mediated gene trap mutagenesis screen designed to detect abnormal mitochondrial phenotypes in mouse embryonic stem (ES) cells, the authors isolated a mutant allele of Sucla2, and these mutant ES cells were used to generate transgenic mice. Animals deficient for Sucla2 exhibited embryonic lethality with the mutant embryos dying late in gestation. Histological analysis of mutant placenta revealed increased mineralization and mutant embryos were found to be approximately 25% smaller than wild-type littersmates. Sucla2 mutant placenta as well as mutant embryonic brain, heart and skeletal muscle showed varying degrees of mtDNA depletion and mutant brains exhibited elevated levels of MMA. SCS-deficient mouse embryonic fibroblasts (MEFs) demonstrated a 50% reduction in mtDNA content compared with normal MEFs. The mtDNA depletion in MEFs and embryonic tissues was revealed to be functionally significant, as it resulted in reduction of steady state levels of mtDNA-encoded proteins, multiple respiratory chain deficiencies, and cellular respiration defects. Furthermore, mtDNA content was restored in mutant cells by reintroduction of Sucla2.
activity via X-gal staining (Friedrich and Soriano, 1991). Staining of e12.5 Sucla2SAβgeo/+ embryos demonstrated strong expression of Sucla2 in the brain, heart, developing spinal cord and/or neighboring tissues with relatively little staining in liver (Fig. 1H). Staining of the e12.5 placenta clearly demonstrated strong expression of Sucla2 (supplementary material Fig. S1). This expression pattern was consistent with previous reports (Lambeth et al., 2004).

Mice homozygous for mutant Sucla2 exhibit late gestational lethality with placental abnormalities

Genetic analysis of progeny from Sucla2SAβgeo/+ heterozygous intercrosses demonstrated that homozygous mutant embryos die late in gestation, predominantly at or after e18.5, with no live born pups identified (Table 1). Western analysis of mouse embryonic fibroblasts (MEFs) established from mutant and wild-type littermate embryos demonstrated a severe reduction of SUCLA2 protein levels associated with a 75% reduction in ADP-specific SCS enzyme activity (Fig. 1F,G; supplementary material Table S2). Interestingly, there was a reciprocal increase in SUCLG2 protein levels that corresponded to a 75% increase in GDP-specific SCS activity that preserved total SCS enzyme activities in Sucla2−/− MEFs. This increase in SUCLG2 protein levels was mediated at a translational or post-translational level, given that there was no detectable increase in Suclg2 transcript levels by quantitative real-time PCR (qRT-PCR; supplementary material Table S3). Ectopic expression of wild-type Sucla2 cDNA in Sucla2−/− MEFs restored SCS activities and SUCLG2 protein expression to wild-type levels, demonstrating the specificity of these phenotypes (Fig. 1F,G; supplementary material Table S2). No structural or developmental defects were identified from histopathological analysis of e17.5 mutant embryos (supplementary material Fig. S2); however, the mutant embryos were on average 25% smaller by weight than littermates (Fig. 2A,B) and their placentas...
exhibited signs of increased mineralization (Fig. 3), suggesting that placental insufficiency might play a pathological role. This was accompanied by a 45% reduction in e17.5 placental mtDNA content by quantitative PCR (qPCR; Fig. 2C; supplementary material Table S4), associated with a trend towards decreased protein levels of COX1, a mtDNA-encoded subunit of cytochrome c oxidase (Fig. 2D).

**Succa2 mutant MEFs demonstrate progressive and functionally significant mtDNA depletion**

Given the histopathological and molecular abnormalities observed in the mutant placentas, **Succa2** mutant MEFs were also examined for potential mtDNA depletion. MEFs derived from mutant e12.5 embryos and grown in uridine-supplemented media exhibited progressive mtDNA depletion in culture compared with MEFs from wild-type littermates (supplementary material Fig. S3). Mutant MEFs demonstrated a 50% depletion of mtDNA after 5 weeks of culture that was rescued by ectopic expression of wild-type **Succa2** cDNA (Fig. 4A; supplementary material Table S4). When histochemically stained for succinate dehydrogenase (SDH, no mtDNA-encoded subunits) activities, both wild-type and mutant cells showed uniform staining for SDH. A proportion (~36%) of mutant MEFs exhibited absent or reduced COX activity, in contrast to wild-type cells demonstrating uniform COX staining (Fig. 4D; supplementary material Fig. S4). Measurement of enzyme activities of individual electron transport chain (ETC) complexes from cell lysates (normalized to citrate synthase activity) showed a significant reduction in complex III and citrate synthase activities, whereas complex II (SDH) activity was mildly increased (Fig. 4E; supplementary material Table S5). The cells were further analyzed by FACS-based analysis of relative mitochondrial membrane potential using the potential-sensitive dye DiIC1(5) (Fig. 1B). After 5 weeks of culture, mutant MEFs demonstrated a relative partial depolarization of the mitochondrial inner membrane (Fig. 4B) in parallel with the mtDNA depletion and ETC deficiencies described above (Fig. 4A,D,E). Furthermore, the cellular respiration of MEFs was analyzed by measuring oxygen consumption when grown in the presence of pyruvate and glucose and sequentially exposed to ETC inhibitors and uncouplers. Oligomycin (an ATPase and complex V inhibitor), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, a mitochondrial uncoupler) and rotenone plus antimycin A (inhibitors of complex I and complex III, respectively) were used in order to measure basal respiration, oligomycin-sensitive respiration (typically reflecting complex V activity) and total respiratory capacity, respectively (Fig. 4C). This analysis demonstrated that the mutant MEFs show significant defects in basal respiration, oligomycin-sensitive respiration and total respiratory capacity (Fig. 4C; supplementary material Fig. S5). In combination, these studies suggest that progressive mtDNA depletion in **Succa2** mutant cells ultimately interferes with proper steady-state expression of mtDNA-encoded subunits, mitochondrial depolarization and perturbation of mitochondrial respiration.

### Table 1. Succa2**−/−** embryos die late in gestation

<table>
<thead>
<tr>
<th>Age</th>
<th><strong>Succa2</strong> genotype</th>
<th><strong>+/+</strong></th>
<th><strong>−/+</strong></th>
<th><strong>−/−</strong></th>
<th>Total*</th>
<th>Chi-squared test²</th>
</tr>
</thead>
<tbody>
<tr>
<td>e9.5-e16.5</td>
<td></td>
<td>50</td>
<td>81</td>
<td>36</td>
<td>167</td>
<td>0.29</td>
</tr>
<tr>
<td>e17.5-e18.5</td>
<td></td>
<td>48</td>
<td>127</td>
<td>33</td>
<td>208</td>
<td>0.0021</td>
</tr>
<tr>
<td>Birth-P2</td>
<td></td>
<td>27</td>
<td>47</td>
<td>0</td>
<td>74</td>
<td>7.28x10⁻⁰⁶</td>
</tr>
</tbody>
</table>

*Total number of animals genotyped from multiple heterozygous intercrosses for indicated age ranges.

²Chi-squared test performed for each age group compared with expected genotype frequencies from equal sample size, assuming wild-type Mendelian ratios.

**Succa2** mutant embryos exhibit progressive and functionally significant mtDNA depletion with elevated levels of MMA

To examine the potential effect of **Succa2** deficiency in other tissues, analysis of mtDNA content in various embryonic tissues was performed. For e15.5 embryos, no mtDNA depletion was detectable in brain, heart, skeletal muscle or liver (Fig. 5A; supplementary material Table S4). In fact, there was a significant increase in mtDNA content in homozygous mutant brain and muscle compared with wild-type littermates. Interestingly, this phenomenon was also detected during the first week of culture of **Succa2** heterozygous and homozygous mutant MEFs grown under various conditions.
In this study, a genetic screen designed to identify genes that, when mutated, confer abnormal mitochondrial phenotypes in cells resulted in the isolation of gene trap allele of Sucla2, the mouse ortholog of the ADP-specific β-isof orm of SCS. Mouse embryos mutant for Sucla2 exhibit deficiency of ADP-specific succinyl-CoA synthetase activity, significant depletion of mtDNA in brain and skeletal muscle and increased cellular content of MMA, which are prominent features observed in patients that have mitochondrial encephalomyopathy with mtDNA depletion associated with SUCLA2 mutations (Elpeleg et al., 2005; Carrozzo et al., 2007; Ostergaard et al., 2007b). Sucla2 mutant MEFs demonstrate functionally significant mtDNA depletion associated with reduced levels of mtDNA-encoded proteins, leading to respiratory chain deficiencies, partial depolarization of the mitochondrial inner membrane and cellular respiration defects. An obvious difference between Sucla2-deficient mice and SUCLA2 patients is that Sucla2−/− mice experience late gestational embryonic lethality, whereas human patients (including patients with homozygous frameshift mutations) appear normal at birth following an uneventful pregnancy, develop symptoms during infancy and typically succumb to their disease during childhood. In contrast, the Sucla2 mutant embryos are smaller than wild-type littermates and their placentas exhibit increased mineralization and mtDNA depletion, which might cause placental insufficiency or dysfunction contributing to embryonic lethality. In humans, mineralization (or calcification) of placenta at term gestation (i.e. after 36 weeks gestation) is considered normal, as placental calcium content increases with gestational age (Poggi et al., 2001). However, significant placental calcification prior to 36 weeks is typically not normal and has been linked to pregnancy-induced hypertension and fetal growth restriction (Chen et al., 2012). In addition, villous trophoblastic basement membrane (TBM) calcifications are associated with congenital disorders and fetal thrombotic vasculopathy (Chen et al., 2012). Increased preterm placental calcification is considered to be a predictor of poor uteroplacental blood flow and adverse pregnancy outcomes (Chen et al., 2012). Increased placental calcification could reflect abnormalities in fetal calcium utilization and excretion and/or impaired calcium metabolism resulting in hypoxic stress in pre-eclamptic syncytiotrophoblasts (Yang et al., 2013). Sucla2 mutant placentas show coarse calcifications that appear to be within spongiotrophoblasts (Fig. 3), rather than the maternal blood space, as seen in the case of human preterm placental calcifications. Because mitochondrial function and energy metabolism are important for cellular calcium handling (Nunnari and Suomalainen, 2012), it is possible that Sucla2 deficiency and energy metabolism dysfunction alters murine placental calcium handling and metabolism, resulting in placental calcification. Currently, it is unclear whether the placental calcifications are cell autonomous consequences of Sucla2 deficiency in the placenta and/or the result of decreased uteroplacental blood flow.

Although human and murine placentas are similar in many aspects, there are distinct anatomical and cellular differences that might make murine placentas more susceptible to SCS deficiency (Malassîné et al., 2003). Human and mouse placenta differ from each other in terms of morphogenesis. A definitive structure of placenta is observed as early as day 21 of pregnancy in humans whereas in mouse a definitive structure is not apparent until midway through gestation (Malassîné et al., 2003). This shortened period of placental maturity relative to the gestational period could render the mouse placenta particularly susceptible to stress induced by defective utilization of nutrients and/or impaired calcium metabolism in the context of mitochondrial dysfunction. In addition, the giant trophoblastic cells of mouse are not analogous to their human counterparts. Mouse giant cells are generated by endoreplication (Soares et al., 1996), which is not the case in
of MMA per se significantly contributes to the late gestational lethality (Peters et al., 2003). Therefore, it is unlikely that toxicity from elevated levels of MMA, but are indistinguishable from wild-type littermates deficient for methylmalonyl-CoA mutase exhibit extremely high levels of MMA, whereas a subset of mutant MEFs exhibit little or no detectable COX activity, in contrast to wild-type cells demonstrating uniform staining for SDH, whereas a subset of mutant MEFs exhibit little or no detectable COX activity, in contrast to wild-type cells demonstrating uniform normal staining.

![Image](https://via.placeholder.com/150)

**Fig. 4.** Sucla2-deficient MEFs exhibit functionally significant mtDNA depletion associated with relative mitochondrial depolarization, cellular respiration defects and respiratory chain deficiencies. (A) Sucla2 mutant MEFs exhibit mtDNA depletion compared with MEFs from wild-type littermates that is rescued by ectopic expression of wild-type Sucla2 cDNA. (B) The relative mitochondrial membrane potential for Sucla2 MEFs was determined by staining cells with DiIC1(5) (HIDC) followed by FACS analysis (three independent lines for each genotype). The graph on the left shows the analysis after 5 weeks of culture with multiple passages. The mutant MEFs demonstrate a progressive relative depolarization of the mitochondrial inner membrane. (C) Cellular respiration analysis of Sucla2 MEFs demonstrate that Sucla2−/− cells exhibit defects in basal respiration, oligomycin-sensitive respiration and respiratory capacity. (D) Histochemical staining of MEFs reveals complex IV deficiency in a subset of Sucla2−/− cells. MEFs were stained for SDH (no mtDNA-encoded subunits) and COX (has mtDNA encoded subunits) activities. All cells show uniform staining for SDH, whereas a subset of mutant MEFs exhibit little or no detectable COX activity, in contrast to wild-type cells demonstrating uniform normal staining. (E) Analysis of mitochondrial electron transport chain enzyme activities shows partial deficiency of ETC complex III and a reduction in citrate synthase (CS) activity in Sucla2 mutant MEFs. CS is a TCA cycle enzyme commonly used as a biochemical marker of mitochondrial matrix content.

What is the mechanism for mtDNA depletion in Sucla2 deficiency?

How deficiency of Sucla2 (SUCLA2) leads to mtDNA depletion is currently not well understood. Previous studies have suggested that SCS forms a complex with a mitochondrial isoform of nucleotide diphosphate kinase (NDPK) (Kavanaugh-Black et al., 1994; Kowluru et al., 2002). In addition, knockdown of SUCLG2 in SUCL2-deficient fibroblasts reportedly results in mtDNA depletion and reduction in NDPK activity (Miller et al., 2011), suggesting that disruption of a SCS-NDPK complex leads to a perturbation of mitochondrial nucleotide (dNTP) pools that affects mtDNA replication. Perturbation of mitochondrial nucleotide pools associated with mtDNA depletion has been demonstrated in cellular and animal models of thymidine phosphorylase deficiency (López et al., 2009; González-Vioque et al., 2011). Deficiency of Sucla2 results in a severe reduction in ADP-specific SCS activity and a reciprocal increase in GDP-specific SCS activity in MEFs (Fig. 1G). Because substrate-level phosphorylation of GDP by SCS is the only source of metabolically generated GTP in the mitochondrial matrix, changes in GDP-specific SCS activity could result in perturbations of mitochondrial GTP content. Therefore, altered ADP- and GDP-specific activities could directly affect mtDNA replication or might have broader regulatory effects, much like those demonstrated with glucose-stimulated insulin secretion in an insulinoma cell line and isolated rat islet cells (Kibbey et al., 2007). Alternatively, SUCLA2 might be a component of the mtDNA nucleoid; loss of nucleoid components can lead to missegregation of mtDNA and loss of mtDNA copy number, as has been demonstrated in yeast lacking another TCA cycle enzyme, aconitate (Chen et al., 2005). Further studies will be required to address the potential mechanisms of mtDNA depletion with Sucla2 deficiency.

**Gene trap mutagenesis and FACS in ES cells is an effective strategy for identifying genes important for mitochondrial function**

The genetic screen described in this report, utilizing gene trap mutagenesis and FACS for surrogate mitochondrial fluorescence markers, was designed to identify genes that when mutated cause abnormal mitochondrial phenotypes. The isolation of a mouse ES cell clone with a mutation in Sucla2, a known mitochondrial disease gene, validates the utility of this approach. It is important to note that the isolated gene trap ES cell clones are at most haploinsufficient for mutated loci, whereas mutations in known nuclear-encoded mitochondrial disease genes are typically recessive (Graham, 2012). This suggests that the surrogate fluorescence markers for mitochondrial mass and mitochondrial membrane potential can
detect subtle phenotypes in cells with heterozygous mutations in genes that cause recessive phenotypes, as described here for *Sucla2* (Fig. 1D). In fact, the presence of subtle phenotypes in heterozygous mutant *Sucla2* cells is also suggested by the detection of increased relative mtDNA content (compared with wild type) in early passage *Sucla2*<sup>−/−</sup> MEFs (supplementary material Fig. S3) and e15.5 *Sucla2*<sup>−/−</sup> brain and muscle (Fig. 5A; supplementary material Table S4). This phenomenon could reflect a compensatory response to loss of SCS activity and/or possible perturbation of mitochondrial nucleotide pools that ultimately fails, resulting in progressive mtDNA depletion.

This genetic screen identified over 20 genes that are involved in a wide array of cellular processes, including transcriptional regulation, post-transcriptional regulation, chromatin modulation, signal transduction and metabolism (supplementary material Table S1). In 2010, Yoon et al. described a RNAi screen in cultured C2C12 cells that utilized a similar surrogate fluorescence mitochondrial marker strategy from which over 150 genes involved in a comparably diverse array of biological processes were identified (Yoon et al., 2010). Interestingly, only *Smarcadi1* was identified in both screens, which could be due to inherent differences in the screen designs, including cell type (ES cell versus C2C12 muscle cell), form of mutagenesis (gene trap versus RNAi), inherent nonsaturating nature of the screens and thresholds for the reproducible change in marker fluorescence. A distinct advantage of performing a genetic screen in mouse ES cells is that transgenic animals can be generated for organismal studies using mutant ES cells, as described in this report. In summary, screening for genes important for mitochondrial function by utilizing gene trap mutagenesis and FACS in mouse ES cells is an effective approach that offers the potential to generate novel animal models and to identify genes that might not be identified from screens in other cell types.

**MATERIALS AND METHODS**

**Gene trap mutagenesis of mouse ES cells**

Mouse ES cells were maintained and grown on mitomycin C-inactivated MEFs (STO cells) in the presence of LIF using standard ES cell techniques (Conner, 2001). AB2.2 129SvEv mouse ES cells were stably transfected with a mitochondrial-targeted EYFP construct (pMito-EYFP, Clontech) that was modified by exchanging the Neo<sup>+</sup> cassette for Puro<sup>+</sup> cassette using standard PCR cloning techniques. Individual Puro<sup>+</sup> ES cell clones were individually analyzed by fluorescence microscopy and FACS for YFP fluorescence. The clones that reproducibly demonstrated high, uniform mitochondrial fluorescence were chosen for gene trapping experiments. The ROSA<sup>βgeo</sup> gene trap construct was transfected into the GP+E86 packaging cell line to derive a retroviral producer cell line; virus titers were determined; and ES cell infections were performed as previously described (Friedrich and Soriano, 1991). ES cell infections were performed using a multiplicity of infection (MOI) of 0.1 to reduce the possibility of multiple integrations per cell.

**Cell sorting of transduced ES cells**

Twenty-four hours after retroviral infection and prior to drug selection, ES cells were stained with 100 nM DiIC<sub>1(5)</sub> (Invitrogen) for 30 minutes at 37°C, 5% CO<sub>2</sub>. AB2.2 129SvEv mouse ES cells were stably transfected with a mitochondrial-targeted EYFP construct (pMito-EYFP, Clontech) that was modified by exchanging the Neo<sup>+</sup> cassette for Puro<sup>+</sup> cassette using standard PCR cloning techniques. Individual Puro<sup>+</sup> ES cell clones were individually analyzed by fluorescence microscopy and FACS for YFP fluorescence. The clones that reproducibly demonstrated high, uniform mitochondrial fluorescence were chosen for gene trapping experiments. The ROSA<sup>βgeo</sup> gene trap construct was transfected into the GP+E86 packaging cell line to derive a retroviral producer cell line; virus titers were determined; and ES cell infections were performed as previously described (Friedrich and Soriano, 1991). ES cell infections were performed using a multiplicity of infection (MOI) of 0.1 to reduce the possibility of multiple integrations per cell.
selection (100 μg/ml) the next day. After 10-14 days of selection, 379 individual clones were picked [98 clones screened for changes in YFP fluorescence and 281 clones screened for changes in DiIC(5) fluorescence], established and subsequently analyzed for stable changes in YFP fluorescence by FACS analysis. The vast majority of identified clones (45/47) demonstrated a stable increase in YFP fluorescence, suggesting an increase in mitochondrial mass.

**Inverse PCR to identify gene trap genomic insertion sites**

To identify the genomic insertion site of gene trap ES cell clones, inverse PCR was performed. ES cell clones were grown in individual wells in a 96-well plate format and genomic DNA was isolated in the plate as previously described (Graham et al., 1997). Approximately 1 μg of genomic DNA was subsequently digested with 10 units of HindIII, Espl or PvuI, and then purified over a Qiagen QIAquick spin column. The recovered DNA was then subjected to intramolecular ligation by adding 10 units of T4 ligase in 50 μl total volume. After the ligation, the DNA was precipitated in the presence of 300 mM sodium acetate and ethanol, washed in 70% ethanol and the pellet resuspended in TE buffer. The isolated ligated DNA was then subjected to nested PCR reactions using the following ROSA-specific primers: for the first round of PCR, PreUSSP (5′-ACCAATCAGTCT-GCTTCTCG-3′) and PreSASprev (5′-CCAGGGTTCCTTGATGATG-3′); for the second round of PCR, USSP (5′-GAGACCCCTCTCAAGGACAGC-3′) and SASPrev (5′-CAAACCTTCCTGCGGCTTCT-3′). DNA fragments amplified by nested PCR were then analyzed by agarose gel electrophoresis, purified and sequenced to determine genomic DNA insertion site (supplementary material Table S1).

**Generation of transgenic animals**

To generate transgenic animals, cells from the Sucla2geo/+ ES cell clone (derived from AB2.2 129SvEsv ES cell line) were microinjected into C57BL/6 blastocysts to generate chimeras. Procedures were carried out by the Baylor College of Medicine Genetically Engineered Mouse Core Laboratory using standard protocols. Germline-transmitting male chimeras were bred with C57BL/6 females to establish the mouse line and all studies therefore were performed using mice on a 129SvEsv/C57BL/6 mixed genetic background. All animal experiments performed conformed to protocols approved by the Baylor College of Medicine IACUC.

**Genotyping and RT-PCR**

Sucla2 mice were genotyped by multiplex PCR using a common forward primer (Sucla2F), and allele-specific reverse primers for wild-type (Sucla2R), and gene trap (ROSABgeoR) alleles (all primer sequences are available on request). PCR products were subjected to agarose gel electrophoresis and the wild-type and mutant bands were identified on the basis of size (wild-type, 963 bp; mutant, 1073 bp). Reverse transcription of 1 μg of RNA was performed using the iScript cDNA synthesis kit (Bio-Rad). A common forward Sucla2 exon primer (Sucla2E2F or ‘E’ in Fig. 1C) and allele-specific reverse primers for wild-type (Sucla2E2R or ‘R’ in Fig. 1C) and gene trap (BgeoR or ‘G’ in Fig. 1C) alleles were used for allele-specific PCR reactions to generate 566-bp wild-type and 500-bp gene trap PCR products, respectively.

**Histology**

Embryos and placentas were fixed in 10% neutral buffered formalin prior to weighing and dissection. No gross external or internal malformations were identified. Tissue samples from major organs and the placenta were routinely processed and paraffin embedded. Paraffin sections (3 micron) were cut and stained with hematoxylin and eosin (H&E). Duplicated 3-micron sections were stained for iron (Prussian Blue reaction) and calcium (von Kossa’s silver nitrate reaction). A separate 3-micron section was stained for calcium with Alizarin Red. Stained tissue sections were pictured using Nikon Eclipse 90i microscope and NIS-Elements software from Nikon.

**Western blotting**

Whole cell lysates from tissues or MEFs were prepared in standard RIPA buffer. After centrifugation of the lysate, soluble proteins were isolated in the supernatant and protein concentration determined according to the Bradford-Lowry method. Protein samples were separated on a 10% SDS-polyacrylamide gradient mini-gel. Proteins were transferred electrophoretically to 0.45-μm polyvinyllidine difluoride membrane for 75 minutes at 100 V. Membranes were blocked for 3 hours in 5% milk-PBS and incubated overnight with antibodies. After three washes with PBS containing 0.05% Tween 20, the membranes were incubated for 2 hours with horsedarish-peroxidase-conjugated goat anti-rabbit or goat anti-mouse (Bio-Rad) diluted in 5% milk-PBS. The secondary antibody was detected using the chemiluminescent ECL Plus reagent (Millipore). Band intensities from autoradiographs were quantified using NIH ImageJ software. Primary polyclonal antibodies used were rabbit α-Sucla2 (1:200; Santa Cruz Biotechnology), rabbit α-Suclg2 (1:10,000; Gene Tex) and rabbit α-Suclg2 (1:10,000; Gene Tex). Primary mouse monoclonal antibodies used were α-COXI (1:1000; MitoSciences) and α-GAPDH (1:100,000; Gene Tex) as loading control.

**X-gal staining**

e12.5 embryos from a cross of Sucla2geo/+ and Sucla2SAgeo/+ mice were isolated and washed with PBS containing 2 mM MgCl2, to remove any traces of blood. Embryos and placenta were then fixed in 4% paraformaldehyde for 2 hours and washed with PBS containing 2 mM MgCl2. Embryos were incubated with X-gal reaction buffer (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl2, 0.02% Nonidet P-40, 0.1% Na deoxycholate and 1 mg/ml X-gal) overnight at 37°C, washed with PBS containing 2 mM MgCl2 and dehydrated. Embryos were briefly incubated in methyl salicylate (Sigma) to clear the tissue and then photographed.

**Cell culture**

MEFs were generated from e12.5 embryos. The embryonic sac was separated from fetal material and cell suspensions were generated from a small portion of embryo (avoiding organs, head and limbs) using 10 mg/ml collagenase H. A portion of whole-cell suspension was taken for genotyping and the rest was plated in a 96-well plate in embryo fibroblast culture medium (cell culture media compositions are available on request). For genetic rescue of Sucla2−− MEf cell line, the full-length Sucla2 cDNA was subcloned into pINDUCER (Meerbrey et al., 2011) that was modified by exchanging the NeoR cassette for PuroR. The Sucla2−PINDUCER (PuroR) construct was stably transfected into Sucla2−− MEFs by electroporation. Ectopic expression of Sucla2 was induced by exposing cells to 100 ng/ml doxycycline for a minimum of 72 hours.

**Mitochondrial membrane potential measurement**

Cells used for mitochondrial membrane potential (MMP) measurement were plated in a six-well plate and DiIC(5) (Invitrogen) was added to a final concentration of 50 nM. The cells were then incubated at 37°C, 5% CO2 for 30 minutes. In parallel, wild-type cells were treated with 50 μM CCCP (Sigma) and 100 nM nigericin (Sigma) at 37°C, 5% CO2, for 30 minutes as controls for depolarized and hyperpolarized mitochondria, respectively. Cells were harvested and then analyzed on a LSRII flow cytometer with 633 nm excitation using emission filters appropriate for Alexa-Fluor-633 dye. FlowJo software was used to analyze the data. At the concentration of nigericin used, a small proportion of the wild-type cells exhibited uncoupling (Fig. 1B), probably due to drug toxicity.

**qPCR and qRT-PCR**

Relative mtDNA content of the MEFs and various tissues were analyzed using real-time qPCR, as described before (Bai and Wong, 2004) with the following modifications. The β2 microglobulin gene (B2M) was used as the nuclear gene (nDNA) normalizer for calculation of the mtDNA/nDNA ratio. The ND1 region of mouse mtDNA was amplified using forward primer, ND1F, and reverse primer, ND1R, giving an amplicon of 160 bp. A fragment of B2M gene was amplified using forward primer, B2MF, and reverse primer, B2MR, giving an amplicon of 106 bp. The relative mtDNA content (mtDNA/B2M ratio) was calculated using the formula: mtDNA content = 1/2ΔCt, where ΔCt = CtB2M-CtB2M. RNA was isolated from three different wild-type and Sucla2 mutant cell lines. Reverse transcription of
1μg of RNA was performed using the iScript cDNA synthesis kit (Bio-Rad) and the cDNA was used to perform qRT-PCR. The fold change of SCS components was measured using the ΔΔCt method.

**Cellular respiration assay**

The XF24 extracellular flux analyzer (Seahorse Biosciences) was used to measure the rates of MEF oxygen consumption. Cells were plated the day prior to the experiment on XF24 cell culture 24-well microplates at a density of 60,000 cells per well. XF assay media (5 mM glucose, 2 mM pyruvate in unbuffered DMEM; Seahorse Biosciences) was prepared and the pH adjusted to 7.0 on the day of the experiment. XF assay media was used to prepare cellular stress reagents: 500 nM oligomycin, 500 nM FCCP, 100 nM antimycin A and 100 nM rotenone (final concentrations). All the reagents were loaded into the injection ports as recommended by Seahorse Biosciences. Oxygen consumption rates (OCR) were cyclically measured with each of the 12 cycles consisting of 3 minutes mixing, 2 minutes equilibration and 3 minutes OCR measurements. After the assay was completed, viable cells in each well were counted using a Vi-Cell XR cell counter and the cell counts used to normalize the OCR rates, with OCR being expressed as pmoles oxygen/minute/10⁶ cells.

**ETC and SCS enzyme assays**

Enzymatic assays of respiratory electron transport chain (ETC) complexes I-IV and citrate synthase were performed as described before (Graham et al., 2010). Briefly, 25 mg of tissue or 10⁶ cells. Briefly, complex I activity (NADH:ubiquinone oxidoreductase) was determined by measuring the oxidation of NADH at 340 nm (using ferriyanide as the electron acceptor). Complex II activity (succinate dehydrogenase) was determined by measuring the reduction of the artificial electron acceptor 2,6-dichlorophenol-indophenol (DCIP) at 600 nm. Complex III activity (ubiquinol:cytochrome c oxidoreductase) was determined by measuring the reduction of cytochrome c at 550 nm. Complex IV activity (cytochrome c oxidase) was determined by measuring the oxidation of cytochrome c at 550 nm. Citrate synthase activity was determined by measuring the reduction of the artificial electron acceptor 2,6-dichlorophenol-indophenol (DCIP) at 600 nm. Complex III activity (ubiquinol:cytochrome c oxidoreductase) was determined by measuring the reduction of cytochrome c at 550 nm. It is coupled to the reduction of acetyl-CoA by citrate synthase in the presence of oxaloacetate. Details of the reaction mixtures are available on request.

Succinyl-CoA synthetase (SCS) activity was measured at 30°C in whole cell lysates from tissues or MEFs in the direction of the succinate to fumarate reaction, as previously described (Lambeth et al., 2004) with some modifications. The complete assay mixture in a volume of 175 μl contained 50 mM potassium phosphate, pH 7.2, 10 mM MgCl₂, 0.2 mM succinyl-CoA, 2 mM ADP (for A-SCS) or 1 mM GDP (for G-SCS), and 0.2 mM DTNB. The reactions were initiated by adding succinyl-CoA and DTNB in quick succession to the above mixture along with cell lysates containing 5 μg of protein. Rates were corrected by subtracting the rate observed in the absence of ADP or GDP. The release of CoA-SH from succinyl-CoA was measured spectrophotometrically at 412 nm, indicating the formation of thionitrobenzoate from the interaction of CoA-SH with DTNB. All activities were calculated as nmoles/milligram protein and expressed as a percentage of control activity.

**Histochemical staining of COX and SDH activities**

Cells to be stained for COX or SDH activity were grown on sterile glass coverslips overnight. Growth medium was removed from the cells and the culture dish placed onto a bed of ice-water slurry. The cells then rinsed three times with ice-cold PBS and once with double-distilled H₂O, and all traces of fluid removed. Cells were air-dried for 3 minutes and then incubated at 37°C with freshly prepared staining buffer. Histochemical staining was performed as previously described (De Paepe et al., 2009) with some modifications. For COX, the staining buffer consisted of 10% sucrose, 100 μM of fully reduced bovine cytochrome C, 8 units catalase, 1 mg/ml 3,3-diaminobenzidine (DAB) and 0.25% DMSO in 20 mM sodium phosphate buffer (pH 7). Cells to be stained for SDH activity were incubated in 0.1 M phosphate buffer (pH 7) containing 1.5 mM nitroblue tetrazolium, 130 mM sodium succinate, 0.2 mM phenazine methosulfate and 1.0 mM sodium azide for 1-2 hours at 37°C. After staining, the cells were rinsed once with 20 mM phosphate buffer, and once with ice-cold methanol, then re-hydrated and mounted in Vectashield. Cells were imaged using a Nikon Eclipse 90i microscope and NIS-Elements software from Nikon. Each cover slip was divided into quadrants and the number of cells (total and positive for staining) were counted manually. Cells positive for staining were expressed as percentage of total cells. Standard deviation was calculated by Student’s t-test.

**Measurement of MMA**

Tissue content of MMA was determined by liquid chromatography combined with tandem mass spectrometry (HPLC-MS/MS) using a method modified from previous publications (Kushnir et al., 2001; Schmedes and Brandslund, 2006). Briefly, 500 μl of tissue extract was mixed with 90 μl of concentrated phosphoric acid and then extracted with 3 ml of tert-butylmethyl ether (MTBE). Next, the dried supernatant was derivatized with butanoic HCl at 65°C for 20 minutes. The analysis was performed on a Waters 2695 Alliance HPLC system connected to a Micromass Quatro Micro tandem mass spectrometer. The column (Waters Symmetry C8, 3.5 μm, 2.1×100 mm) was eluted isocratically with 85% methanol containing 5 mM ammonium formate. Positive ion mode was used. The multiple-reaction monitoring selected for MMA and d-MMA were m/z 231>119 and 234>122, respectively. Nebuliser gas flow was set to 20 l/hour and desolvation gas 580 l/hour. Cone voltage was 35 V; capillary 3800 V and collision 10 V. The amount of MMA was normalized to the protein content of the tissue extract.

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**Competing interests**

The authors declare no competing financial interests.

**Author contributions**


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**Supplementary material**

Supplementary material available online at http://dmm.biologists.org/lookup/suppl?doi=10.1242/dmm.013466/-/DC1

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