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The interplay between *SUCLA2*, *SUCLG2* and mitochondrial DNA depletion

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**Title:****The interplay between *SUCLA2*, *SUCLG2* and mitochondrial DNA depletion.**Chaya Miller<sup>1</sup>, Liya Wang<sup>2</sup>, Elsebet Ostergaard<sup>3</sup>, Phyllis Dan<sup>1</sup>, Ann Saada<sup>1\*</sup>

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**Abstract**

*SUCLA2*-related mitochondrial DNA (mtDNA) depletion syndrome is a result of mutations in the  $\beta$  subunit of the ADP-dependent isoform of the Krebs cycle succinyl-CoA synthase (SCS). The mechanism of tissue specificity and mtDNA depletion is elusive but complementation by the GDP-dependent isoform encoded by *SUCLG2*, and the association with mitochondrial nucleoside diphosphate kinase, (NDPK) is a plausible link.

We have investigated this relationship by studying *SUCLA2* deficient fibroblasts derived from patients and detected normal mtDNA content and normal NDPK activity. However, knockdown of *SUCLG2* by shRNA in both patient and control fibroblasts resulted in a significant decrease in mtDNA amount, decreased NDPK and cytochrome *c* oxidase activities, a marked growth impairment. This suggests, that *SUCLG2* to a higher degree than *SUCLA2* is crucial for mtDNA maintenance and that mitochondrial NDPK is involved. Although, results pertain to a cell culture system, the findings might explain the pathomechanism and tissue specificity in mtDNA depletion caused by defective *SUCLA2*.

**Key words**

mtDNA depletion, succinyl-CoA synthase, *SUCLA2*, *SUCLG2*, NDPK, mitochondrial respiratory chain

## 1. Introduction

Mitochondrial DNA depletion syndromes (MDS) are a genetically heterogeneous group of autosomal recessive disorders characterized by decreased mtDNA content with accompanying reduction of the activities of mitochondrial respiratory chain (RC) complexes I,III,IV and V. MDS usually present in infancy or childhood in a tissue specific manner mainly affecting brain and muscle[1,2]. *SUCLA2*-related mitochondrial MDS (MIM ID#612073) is a result of mutations in the *SUCLA2* gene encoding the  $\beta$  subunit of the ADP-dependent isoform of succinyl-CoA synthase (SCS) ( EC 6.2.1.5 )[3] . SCS is a mitochondrial matrix enzyme that catalyzes the reversible synthesis of succinyl-CoA from succinate and CoA. The reverse reaction occurs in the Krebs cycle, while the forward reaction may produce succinyl-CoA for activation of ketone bodies and heme synthesis. SCS is composed of an invariant  $\alpha$  subunit encoded by *SUCLG1* and a  $\beta$  subunit that determines the enzyme's nucleotide specificity. The GTP-specific  $\beta$  subunit encoded by *SUCLG2* is a component of the second SCS isoform (EC 6.2.1.4) which catalyzes GDP dependent reactions[4]. Deficiency of *SUCLA2* results in Leigh or a Leigh-like syndrome with onset of severe hypotonia in early infancy, severe muscular atrophy and sensorineural hearing impairment. Most patients die in childhood. The metabolic findings usually show urinary excretion of methylmalonic acid (MMA), elevated plasma methylmalonic acid concentration, and elevated plasma lactate concentration [5,6,7].

*SUCLG1* deficiency can be more severe than *SUCLA2* deficiency causing MDS with fatal, infantile lactic acidosis (MIM ID#245400)[1,3], but a phenotype indistinguishable from that of patients with *SUCLA2* deficiency has also been described[8,9].

The pathomechanism of SCS deficiency remains elusive as there is no obvious connection between a Krebs cycle enzyme and mtDNA metabolism. A plausible link might be the possible association between the mitochondrial form of nucleoside diphosphate kinase, (NDPK)(*NM23-H4*, *NME4*), and SCS [10,11,12].

We have investigated this association by studying normal fibroblasts and *SUCLA2* deficient fibroblasts derived from patients, and show that knockdown of *SUCLG2* results in a decreased mtDNA content, COX activity and mitochondrial NDPK activity

## 2. Materials and Methods

### 2.1 Tissue culture

Primary fibroblasts (from forearm biopsies, taken with informed consent, approved by the local IRB) were obtained from three patients. Patient ELN, harbored a complex genomic rearrangement in the *SUCLA2* gene, with a homozygous deletion of 43 nt, encompassing the last 14 nt of exon 6 and the first 29 nt of IVS6, and an insertion of 5 nt (32720del43ins5). This mutation was reported to result in the skipping of exon 6 [5]. RJ harboured as homozygous G-to-A transition in intron 4 of the *SUCLA2* gene (534+1G>A) reported to result in the skipping of exon 4 [6]. In patient AK, a deletion of the whole gene was found. None of the exons could be amplified by PCR, and a homozygous deletion of the gene was confirmed by quantitative PCR of the first and last exons. The cells were cultured in DMEM medium containing 4.5g glucose/l and supplemented with 10% fetal calf serum, 50 ug/ul uridine and 110 ug/ml pyruvate (permissive growth medium). For growth assessment,  $12 \times 10^3$  cells were seeded in triplicates on 24 well plates. The following day the medium was changed to the usual medium or to glucose-free RPMI supplemented with 10% dialysed fetal calf serum and 5mM galactose (GAL medium). After 72 hr, viable cells were counted by trypan blue exclusion. All other measurements were performed in cells obtained from confluent tissue cultures. (All reagents for tissue culture were from Biological Industries Kibbutz Beit Haemek Israel).

### 2.2 shRNA interference

A mixture of MISSION® shRNA plasmid DNA vector targeted against *SUCLG2* or a nontarget shRNA control vector (Sigma Aldrich) were introduced into 293FT cells by cotransfection with pLP1, pLP2 and pLP/VSVG plasmids using lipofectamine according to the manufacturer's instructions (ViraPower, Invitrogen, California, USA). The virus-containing supernatants were harvested, filtered, and target cells were infected with virus supernatant containing polybrene. Stably transfected cells were obtained by selection with puromycin (4 mg/ml).

### 2.3 mRNA transcript analysis

RT-PCR was performed in order to study the effect of *SUCLA2* mutations on exon skipping and to show down regulation of *SUCLG2* following shRNA.

Total RNA from fibroblast was prepared using TRI reagent (Sigma-Aldrich) and equal amounts were reverse transcribed using ImPromII (Promega, Wisconsin, USA) reverse transcriptase kit with a hexamer mixture as the template primer. PCR, (*SUCLA2*, *SUCLG2* and  $\beta$ -actin) was performed on cDNA samples using the following primers:

*SUCLA2* forward – 5' TGTCGTGATAAAGGCACAGG 3'

*SUCLA2* reverse – 5' CTTTTGGCGATAGGCTGAAT 3'

*SUCLG2* forward -5' CCCTAATGTTGTGGGACAGC 3'

*SUCLG2* reverse – 5' GGAATTCTGCGTTGTCATCA 3'

$\beta$ -actin served as an intrinsic control for *SUCLG2* using the following primers

forward – 5' CGAGGCCCCCTGAAC 3'

reverse – 5' TCTCAAACATGATCTGGGTCATCT 3'

An approximate estimation of the reduction *SUCLG2* transcripts induced by shRNA, was obtained by digital imaging with TINA2 software.

### 2.4 mtDNA quantification

Total mtDNA was quantified by real-time PCR essentially as described [13].

The primer pair for mtDNA was 5'-GTACCCACGTAAAGACGTTAGG-3' and 5'-TACTGCTAAATCCACCTTCG-3'. The fluorescent probe CCCATGAGGTGGCAAGAAAT was labelled at the 5'-end with the reporter dye VIC and at the 3'-end with the quencher dye TAMRA. RNaseP served as a nuclear DNA control. Cycling and fluorescence measurements were performed by an ABI Prism 7700 Sequence Detection System (Applied Biosystems, USA).

### 2.5 Mitochondrial preparation and enzymatic analyses

Mitoplasts were prepared from ca  $4 \times 10^6$  cells by homogenization, centrifugation and digitonin treatment [14]. Enzymatic activity of cytochrome *c* oxidase (COX) was measured by following the oxidation of 50  $\mu$ M reduced cytochrome *c* at 550nm in the presence of 10mM potassium phosphate buffer with pH 7.0 at 37° C.

NDPK activity was assayed with 10  $\mu\text{M}$   $^3\text{H}$ -TDP as the labeled substrate in 20 mM Tris/HCl, pH 7.6, 3 mM DTT, 1 mM  $\text{MgCl}_2$  and 0.2 mM ATP. Reaction was started by addition of extracts/enzyme, incubated at 37°C for 3 min and stopped by addition of EDTA and kept on ice. Ten  $\mu\text{l}$  of reaction mixture was applied to TLC (PEI-cellulose, MERCK). The reaction products were then separated by 0.2 M  $\text{NaH}_2\text{PO}_4$ . Non-radioactive nucleotides were used as marker. The products was cut out, eluted, mixed with scintillation fluid and counted. The  $^3\text{H}$ -TDP, used for NDPK activity measurements, was synthesized enzymatically from  $^3\text{H}$ -dThd (methyl- $^3\text{H}$ -thymidine, Perkin Elmer) using Uu-TK and Ba-TMPK and purified [15,16]. The enzymatic activities were normalized to citrate synthase (CS), a mitochondrial control enzyme which was measured spectrophotometrically as described[17].

### 2.6 Immunocytochemistry

Fibroblasts grown on fibronectin-coated coverslips were incubated with MitoTracker Red CM-H<sub>2</sub>XRos (Molecular probes, Eugene, Oregon USA), fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, incubated with primary antibodies directed against TFAM (a generous gift from Dr. Manuel Rojo, Bordeaux, France), detected with FITC conjugated secondary antibodies (Jackson Immuno Research laboratories West Grove, PA, USA) and visualized by confocal microscopy (10 x 40). For measuring DNA synthesis, fibroblasts were grown for 24h in the presence of 15 $\mu\text{M}$  5-bromo-2-deoxyuridine (BrdU (MP Biomedicals, Solon, OH, USA)) in rich growth medium but without uridine, prior to MitoTracker staining and fixation. The cells were permeabilized with Triton-x100 and the antigen was exposed by 2M HCl and detected by mouse-anti BrdU (AbD Serotec, Oxford UK) and FITC conjugated secondary antibodies. (Jackson Immuno Research laboratories West Grove, PA, USA)

### 3. Results

The disruption of *SUCLA2* mRNA in the patients cells was shown by RT-PCR (Fig 1A). Control fibroblasts showed the expected 567bp fragment including exons 3-6 and part of 7 while the patient samples ELN and RJ disclosed shorter RT-PCR products, corresponding to the expected lengths, of 438bp and 414bp respectively, confirming the presence of truncated transcripts. Patient sample AK did not display any detectable message, as expected (Fig 1A).



Semi quantitative analysis by RT-PCR of the reduction of *SUCLG2* mRNA induced by shRNA treatment, disclosed a significant, 95% decrease in two controls (one is depicted in Fig1B) and a partial decrease, 85%, 60% and 40% in AK, RJ and ELN respectively. Cells treated with non-target control shRNA displayed normal transcript amounts (Fig.1B).

The mtDNA/nDNA ratios were within the normal range in all patient cells (Fig. 2A). The COX activity was near normal in ELN, slightly decreased in RJ, and markedly decreased in AK (Fig 2B) while the NDPK activities were not statistically different from the controls (Fig. 2C). Growth of ELN and RJ was more severely impaired on glucose free media (41% and 64% of normal growth rates, respectively) relative to controls (71% of normal growth rates), while growth of AK was slow in both media.

All *SUCLG2* shRNA treated cells, including controls, displayed a notable, 34%-56% decrease in mtDNA/nDNA ratio compared to their respective control vector treated cells (Fig. 2A). COX activity was also relatively decreased in controls and in ELN (25%), whereas a less significant decrease in cells with an *a priori* lower COX activity was found (Fig.2B). A considerable (45%-60%) decrease in NDPK activity was observed in all *SUCLG2* shRNA treated cells (Fig.2C). Growth on GAL medium was severely affected not only in *SUCLG2* shRNA treated patient cells but also in control cells (32% growth) compared to cells treated with control vector. The decrease in control cell growth was comparable to that of RJ and ELN (41% and 13% respectively). AK cells did not display any growth, but rather detached and the final cell count was less than the initial number seeded. Generally, the growth of shRNA treated cells in rich medium was impaired, but less markedly than in GAL (Fig.2C). These cells also quickly detached and died upon serum starvation while untreated cells remained in stationary state (results not shown).

The defective growth and decreased mtDNA content was also demonstrated by a decreased BrdU incorporation both in the nucleus and in the mitochondria, as well as a decrease of staining of the mitochondrial translation factor TFAM as depicted in a control and in patient ELN (Fig.3).

## 5. Discussion and Conclusions

We hereby show that fibroblasts from patients with *SUCLA2* deficiency have normal amounts of mtDNA, normal NDPK activity, normal or slightly decreased COX activity, and in one cell line an OXPHOS deficiency manifesting as decreased growth in galactose media. Knockdown of *SUCLG2* by shRNA in both patient and control fibroblasts resulted in a significant decrease in mtDNA

content, decreased NDPK activity and a partial decrease in COX activity. Growth was impaired, and most markedly in OXPHOS dependent GAL medium.

The brain and skeletal muscle are the primary organs affected by *SUCLA2* deficiency. We have previously hypothesized that the tissue specificity is caused by the inability of *SUCLG2* to compensate for *SUCLA2* deficiency, as *SUCLG2* is only weakly expressed in brain muscle. Notably, *SUCLG2* is highly expressed in liver and this tissue remains unaffected in patients with *SUCLA2* defects[4,5, 18]. We also speculated that mtDNA depletion in non-dividing tissue in patients with *SUCLA2* mutations is caused by disruption of the normal interaction of *SUCLA2* with the mitochondrial NDPK, leading to imbalanced mitochondrial deoxyribonucleotide (dNTP) metabolism[5,19].

Accordingly we anticipated to find near normal or normal mtDNA content and COX and NDPK activity, in patient cells. The normal mtDNA/nDNA ratio in the patient cells, suggested that *SUCLG2* is indeed able to compensate for the disrupted *SUCLA2* in mtDNA maintenance (Fig. 2A). Nevertheless, the COX activity in patient cells was partially decreased. This partial impairment of the respiratory chain in patient cells was also detected by observing the relative growth impairment in GAL medium (Fig. 2B and D). Notably, growth in glucose free medium is highly dependent on oxidative phosphorylation (OXPHOS) while ATP can be obtained from glycolysis in glucose-containing medium. The OXPHOS dysfunction seems to be due to an mtDNA-independent, secondary mechanism. The unaltered NDPK activities in patient cells also supported this.

By knocking down the *SUCLG2* by shRNA in patient cells we aimed to investigate the role of *SUCLG2* in compensating for *SUCLA2*. The severely decreased mtDNA content, COX activity and OXPHOS-dependent growth supports the hypothesis that *SUCLG2* can compensate for a *SUCLA2* defect. This occurred concomitantly with a significant decrease in NDPK activity stressing the importance of NDPK in mtDNA maintenance.

Regrettably we were not able to accurately measure the amount NDPK protein due to unsatisfactory signal to noise ratio with different antibody preparations.

The impairment of growth, especially OXPHOS dependent growth, in control cells could be attributed to impairment of the Krebs cycle, suggesting that *SUCLG2* is vital in intermediary metabolism and cannot be replaced by *SUCLA2*. However, we had not anticipated that downregulated *SUCLG2* in normal cells would show any effect on mtDNA content or COX and NDPK activities.

The defect in growth and mtDNA content was also clearly visualized by immunocytochemistry. Notably the reciprocal experiment, *SUCLA2* shRNA treated normal cells were not significantly different from the control vector (results not shown).

We conclude and suggest that *SUCLG2* to a higher degree than *SUCLA2* is crucial for mtDNA maintenance and that mitochondrial NDPK is involved.

Our findings also confirm the suggested mechanism for tissue specificity, the more severe phenotype of some *SUCLG1* patients and may also explain the (to our knowledge), lack of *SUCLG2* deficient patients, as such a defect might be incompatible with life.

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**Legend to figures****Fig. 1. Analysis of *SUCLA2* and *SUCLG2* mRNA**

Total RNA from control and patient (AK, RJ, ELN) cells untreated (1A), treated with *SUCLG2* shRNA (sh) or control vector shRNA (cv) (1B). RNA was reverse transcribed to cDNA and subjected to PCR using specific primers to *SUCLA2* or *SUCLG2*.  $\beta$ -actin served as an intrinsic control (1B). Size markers were run in the flanking slots.

**Fig. 2. Enzymatic activity of NDPK and COX, cell growth and mtDNA content in cells treated with *SUCLG2* shRNA**

Fibroblasts from controls (n=2) and patients (AK, RJ, ELN) were stably transfected with shRNA against *SUCLG2* (*SUCLG2*sh) or a control vector (cv).

mtDNA/nDNA ratios are presented as percentage relative to controls transformed with the control vector (A). Enzymatic activities of COX (B) and NDPK (C) in isolated mitoplasts are presented as the ratio to CS. Growth was assessed in rich growth medium and glucose free medium (GAL) by counting viable cells.

Results are presented as mean  $\pm$  SD from duplicate samples, performed on at least two separate occasions.

**Fig. 3. Immunocytochemistry of BrdU incorporation and TFAM in cells treated with *SUCLG2* shRNA**

Fibroblasts from control and patient ELN were stably transfected with shRNA against *SUCLG2* (*SUCLG2*sh) or a control vector (cv).

The upper panel contains cells incubated with BrdU and mitotracker (red) and subsequently stained with anti-BrdU antibodies (green). The lower panel contains cells incubated with mitotracker (red) and subsequently stained with anti-TFAM antibodies (green). The figure depicts the overlay (yellow) as detected by confocal fluorescence microscopy.

Figure1

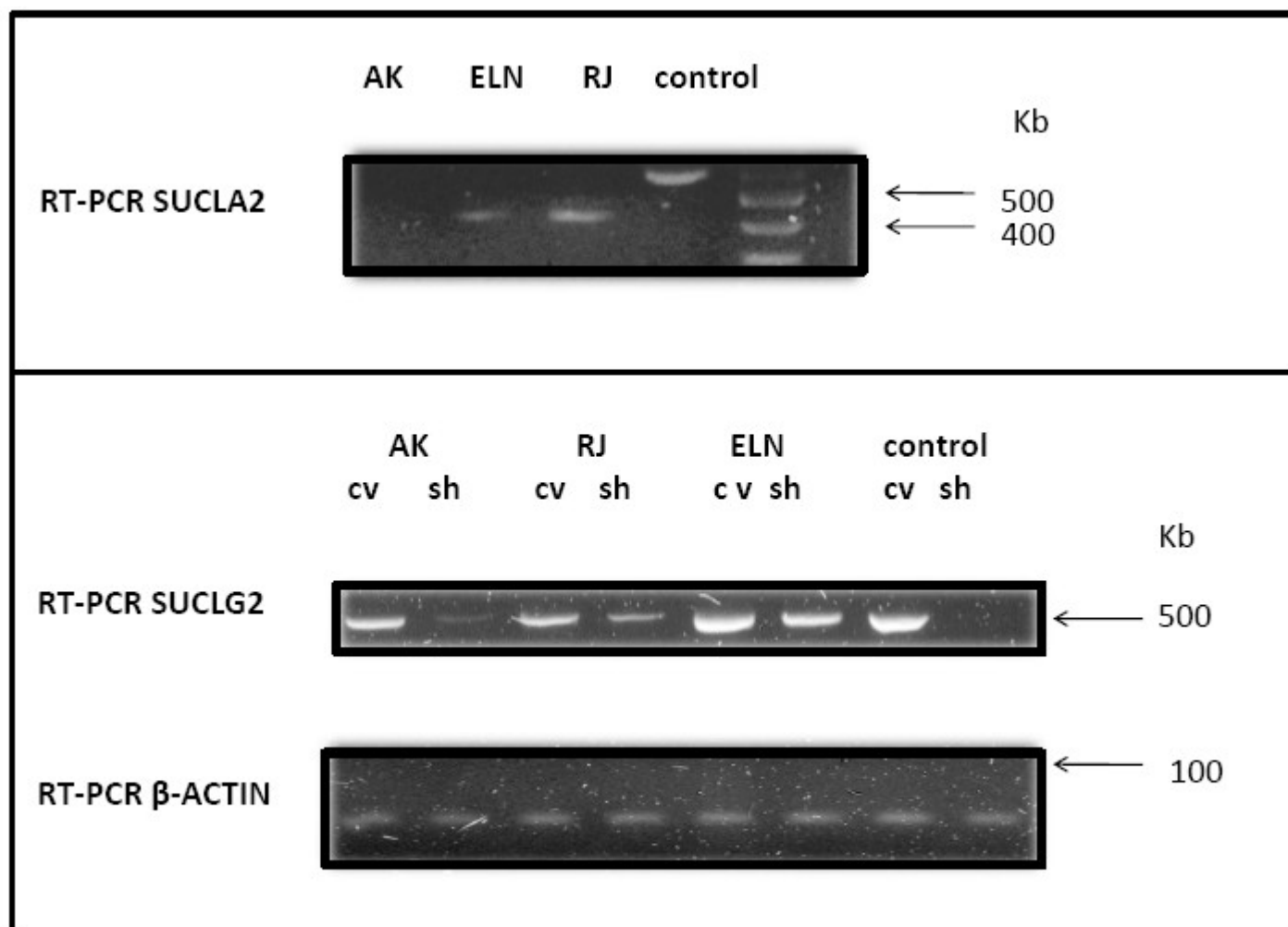


Figure 2

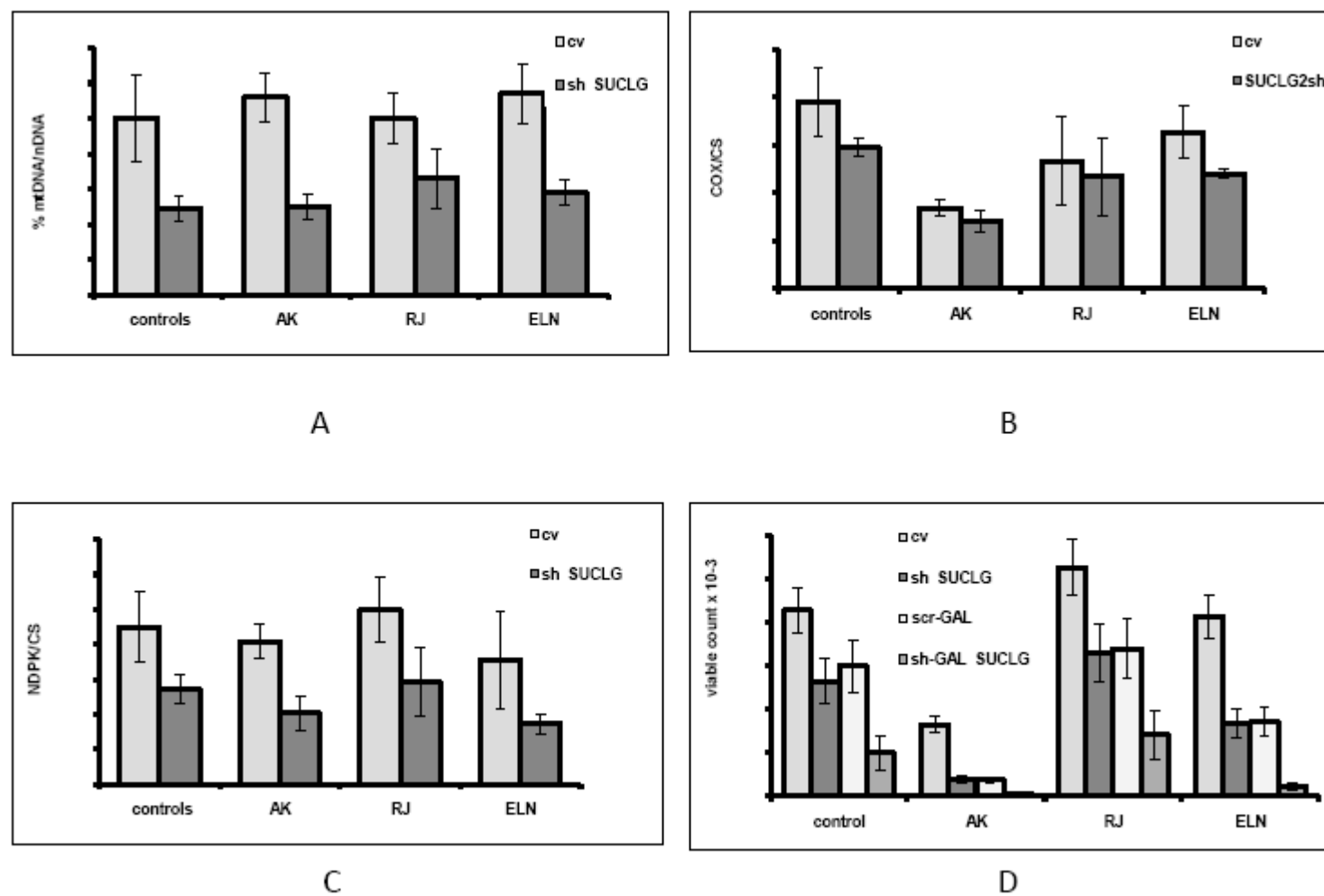




Figure3

