iScience, Volume *23*

Supplemental Information

Electron Transport Chain Complex II Regulates Steroid Metabolism

Himangshu S. Bose, Brendan Marshall, Dilip K. Debnath, Elizabeth W. Perry, and Randy M. Whittal

1 **SUPPLEMENTAL INFORMATION**

Transparent Methods

Cell proliferation and isolation of mitochondria from tissues

MA-10 cells (Mouse Leydig) were cultured in Weymouth media (Sigma, St. Louis, MO) in the presence of 1X L-Glutamine, 10% horse serum and 5% FBS. Cells were kept at 37˚C in a humidified incubator with 5% CO_2 and mitochondria were isolated as described before (Bose et al., 2002). Sheep Adrenal glands, were obtained from the University of Florida, Gainesville, Animal Science Department, immediately after sacrifice. Tissues were diced in an ice-cold mitochondria isolation buffer containing 250mM sucrose (Bose et al., 2008), and mitochondria were isolated using a hand-held, all glass Dounce homogenizer following a previously described protocol (Bose et al., 2008). Most of the experiments were carried out with freshly isolated mitochondria from either cells or tissues. Malate dehydrogenase knockdown in MA-10 cells was performed with the silencing vector from Open Biosystem (Santa Cruz Biotechnology) and the knockdowns were selected with the antibiotic (50 μg/ml) hygomycin. Out of the five plasmids provided by the manufacturer only two plasmids were able to knockdown, as determined by western blotting with MDH2 antibody (Santa Cruz).

Isolation and fractionation of mitochondria

Mitochondria were isolated from mouse testis or sheep adrenals or cultured MA-10 cells by differential centrifugation as described previously (Bose et al., 2008). Mitochondrial pellets were resuspended in a 1:1 mix buffer and kept on ice. Mitoplasts were prepared by solubilizing the outer mitochondrial matrix (OMM) in 1.2 % (w/v) digitonin, a non-ionic detergent. Prior to digitonin treatment, mitochondria were incubated with 10mM HEPES (pH 7.4) for 5 min and centrifuged at 10,000 ×g for 20 min to separate the OMM from the inner mitochondrial matrix (IMM) and matrix (mitoplast). The OMM fraction was centrifuged at 130,000 ×g for 1 h to separate unimported SCC from the pellet membranes. IMM and matrix fractions were prepared by the treatment of mitoplasts with 0.16 mg of non-ionic lubrol (Ragan et al., 1986) per mg of mitochondria followed by ultracentrifugation at 130,000 ×g for 1 h. The soluble fraction was referred to as the matrix and insoluble part as the IMM, although it should be noted that this fraction also contains some intermembrane space (IMS) components. The matrix fraction

(supernatant) was removed, and the membrane pellet was resuspended in 1:1 mix buffer and kept on ice. The volume of the supernatant was measured, and the membrane pellet was resuspended in 1:1 mix buffer.

Western blot

For native Western staining, mitochondria were isolated from rat adrenal or testicular tissues or from the MA-10 cells following our previously described procedure (Pawlak et al., 2011a). The native complex was isolated by incubating mitochondria with buffer containing 1% digitonin, and samples were separated by electrophoresis through 4-16% native gradient gels. For denatured Western blotting, 12.5 mg of total or mitochondrial proteins were loaded per well. After the protein complexes from the native PAGE or SDS-PAGE were transferred to a polyvinylidine difluoride (PVDF) membrane, they were blocked with 3% nonfat dry milk for 45 min, probed overnight with the primary antibodies, and then incubated with peroxidase-conjugated goat anti-rabbit IgG or anti mouse IgG (Pierce, Rockford, IL). Signals were developed with a chemiluminescent reagent (Pierce). For direct visualization of the complexes, the gels were stained with Serva blue or Coomassie blue stain overnight at 4 °C. Unless otherwise indicated, antibodies to P450c11AS, VDAC2, and StAR were all purchased from Santa Cruz Biotechnology (Santa Cruz Biotech, Dallas, TX) or AbCam (Boston, MA). For antibody shift experiment, MA-10 cells were stimulated with cAMP for 18 h and then the mitochondria were isolated as described above. The mitochondrial pellet was solubilized with 1% digitonin, and then specific antibody was added at a previously determined dilution for 60 min. The antibody complex was isolated by centrifugation at 9000 rpm (Beckman Allegra 22XR, F630 rotor) for 45 min, resolved by 4 to 16% native gradient PAGE, transferred to a PVDF membrane, and western blotted with specific antibodies as described previously (Prasad et al., 2017).

***In vitro* synthesis and processing of the precursor protein with different mitochondrial fractions**

The full-length SCC cDNA was subcloned in SP6 vector and the protein was translated in a rabbit reticulocyte transcription translation system in the presence of ^{35}S-labeled methionine following manufacturer's instructions (Promega, Madison, WI). Freshly isolated mitochondria were incubated with a ^{35}S-labeled SCC or mutant SCC at 26˚ C for 1 h or as needed. The imported fractions were separated

from the unimported fraction by extraction with sodium carbonate as described previously (Bose et al., 2002). The membrane integrity was determined by proteolysis with proteinase K as described previously (Bose et al., 2019; Bose et al., 2002).

To confirm that the unimported form is not an aggregate, we performed ultracentrifugation of cell free transcription translation system (CFS) at 144,000 ×g before incubation with mitochondria. Partial proteolysis was performed with PK or trypsin independently with varying concentrations from 10 μg to 250 μg for PK and 1 to 100 Units of sequencing grade trypsin (Thermo Fisher). The import reactions were carried out at 26˚ C. In some experiments import reactions were carried out at different temperatures and also in presence of different inhibitors where Oligomycin 50 μg/mL, valinomycin 2.0 μg/mL and atractloside (CTL) 50 μg/mL were added just prior to start ^{35}S-SCC mitochondrial import. Similarly to study preunfolded ^{35}S-SCC was incubated with 1.0M urea in 10mM Tris buffer pH 7.4 and incubated with the freshly isolated mitochondria from MA-10 cells and import reactions were carried out at 26˚ C for 1 h and terminated by adding mCCCP.

For analysis of the assembled proteins in the complex, mitochondria from the transfected cells or testicular tissues were re-isolated and lysed in digitonin buffer (1% digitonin, 20mM Tris-HCl, pH 7.4, 0.1mM EDTA, 50mM NaCl, 10% glycerol, 1mM phenylmethylsulfonyl fluoride) for 15 min on ice. The digitonin lysate was combined with native-PAGE sample buffer (5% Coomassie Brilliant Blue G-250, 100mM BisTris, pH 7.0, 500mM 6-aminocaproic acid) and subjected to 4–16% or 6-13% gradient native-PAGE at 100 V overnight at 4° C. The native page was stained following electrophoresis to avoid any aberrant complex. Protein complexes were further fractionated by applying 100 μL of digitonin lysate to a 30-10% sucrose density step gradient, with a 200-μL 66% sucrose cushion at the bottom (final volume = 2.0 mL). Following centrifugation at 55,000 × *g* in a Beckman TLA55 rotor for 4 h, the sample was immediately equally aliquoted and loaded onto gradient, native-PAGE or SDS-PAGE gels. Radiolabeled proteins were detected by autoradiography or on a phosphorimager.

Expression kinetics of SCC in COS-1 cells

SCC cDNA was subscloned in the pCMV vector as described previously (Bose et al., 2019), and plasmid cDNA was purified through Qiagen Midi prep columns (Qiagen, Frederick, MD) following the manufacturer's procedure. Briefly, 1×10^6 COS-1 cells were cultured onto a six-well plate 14 h before the transfection, and 2 μg of cDNA was transfected in 3 wells using Lipofectamine (Thermo Fisher, Waltham, MA) as previously described (Bose et al., 2008). Following replacement of medium containing serum and antibiotics, cells were collected in every 4 h, and the SCC expression was determined by Western blotting with a SCC antibody (a kind gift from Dr. Bon-Chu Chung).

Mitochondrial viability analysis

We determined mitochondrial viability isolated from MA-10 cells or from rat testis following a recently developed procedure (Marriott et al., 2012; Prasad et al., 2016). In brief, mitochondrial membrane responsiveness was determined using an ATP Assay System Bioluminescence Detection Kit (ENLITEN, Promega) with a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA) following the manufacturer's protocol. ATP production was inhibited by incubation of MA-10 cells with various concentrations of carbonyl cyanide m-chlorophenyl hydrazone (mCCCP) for 1 h as described previously (Prasad et al., 2017).

Metabolic conversion/Activity assays

To measure the conversion of cholesterol to pregnenolone, isolated mitochondria were incubated with 80,000 counts of ^{14}C-cholesterol (PerkinElmer, Whatman, MA). Reactions were initiated by addition of 50μM NADPH (Sigma-Aldrich) and incubated at 37°C for 2 h in a shaking water bath. For conversion of progesterone analysis the reactions were performed with NADH following our published procedure (Pawlak et al., 2011b; Prasad et al., 2016). To stop pregnenolone to progesterone conversion, we have incubated with 3-beta Hydroxy steroid dehydrogenase inhibitor (3βHSD2) with 10 pmol of Trilostane (Tril) (Prasad et al., 2012). For digitonin solubilized complex activity was performed after isolation of the complex in presence and absence of digitonin and performed the conversion with NADH following the

same procedure. Steroids were extracted following our previous procedure (Pawlak et al., 2011b; Prasad et al., 2017; Prasad et al., 2016) and the amounts of steroid were determined by using a phosphorimager.

Calorimetry

Mitochondria were prepared as described previously and for the specific experiment 0.1 mg of mitochondrial protein was suspended in 1.5 mL of buffer (Bose et al., 2002). The solution was titrated with mitochondria, succinate and ATP in a MicroCal (Northampton, MA) VP-ITC calorimeter equipped with an automatic injector in the Department of Biochemistry and Molecular Biology at the University of Florida, Gainesville. All buffers were filtered through 0.22 μm and degassed before use. Data were collected by incubating mitochondria in the cell for 2 h prior to the injection of succinate or cholesterol in 5 μL at a speed of 0.5 μL/s with a gap of 7 min for each injection for equilibration. Enthalpy (ΔH) was measured by integration of excess power generated by the reaction divided by the concentration of the injectant.

Transmission electron microscopy (TEM)

To determine the precise localization of SCC in mice testis mitochondria, TEM experiments were carried out. Testes tissue was fixed overnight in 4% formaldehyde and 0.2% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.4. Tissues were dehydrated through a graded ethanol series from 25% to 95% and embedded in LR white resin. Sectioning was performed on a Leica EM UC6 ultramicrotome (Leica Microsystems, Bannockburn, IL), and 75nm-thick sections were collected on 200 mesh nickel grids. Sections were etched with 2% H_2O_2, quenched in 1M NH_4CL, and blocked in 0.1% BSA in PBS for 4 h at room temperature in a humidified atmosphere, followed by incubation with anti-SCC (1:1000 dilution) in 0.1% BSA overnight at 4°C. Sections were washed in PBS (5 times) and floated on drops of anti-primary-specific, ultra-small (<1.0 nm) NanogoldTM reagent (Nanoprobes, Yaphank, NY) diluted 1:2000 in 0.1% BSA in PBS at room temperature for 2-4 h. Sections were washed with PBS (5 times, 10 min each) and deionized H_2O (5 times, 10 min each), prior to enhancement with HQ SilverTM (Nanoprobes) for 8 min. Enhancement was terminated by washing in cold deionized H_2O. For double

antibody immunolabeling, sections were labeled with anti-SCC (1:1000) overnight at 4°C, followed by incubation with anti-Calnexin (1:2000) overnight at 4°C. Silver enhancement of anti-SCC nanogold was twice the duration of anti-Calnexin nanogold. Thus, two different sizes of gold particles were obtained. Sections were then stained with 2% uranyl acetate in 70% ethanol, washed with deionized H_2O (5 times, 2 min each), and air dried. Average size of large gold particles was 55 nm in diameter with 90% being between 45-65 nm in diameter. Average size of small gold particles was 15 nm in diameter with 90% of the gold particles being <25 nm in diameter. Tissue sections were imaged using a JEM 1230 transmission electron microscope (JEOL Peabody, MA) at 110 kV and an UltraScan 4000 CCD camera and first Light Digital Camera Controller (Gatan Inc., Pleasanton, CA). Approximately 80 sections from each experiment were analyzed.

Figure preparation and data analysis

The images were obtained from the autoradiographic films or scanning through a phosphorimager, and the data analysis was performed using, Kalidagraph, or Microsoft Excel.

Supplementary Figure Legend

Figure S1 (Related to Figure 1, panels E-H). Enlarged EM of the mouse testis stained with SCC antibody. The distance of mitochondria from the ER is shown in blue arrow.

Figure S2 (Related to Figure 1, panels K-M). Analysis SCC processing. (A), Overexpression of SCC in COS-1 cells at the indicated time detected by Western blotting with a SCC antibody. The red arrow head shows the minimum start of expression and blue arrow head shows start of major conversion from 61 kDa to 51 kDa. Bottom, Western blot of the same fractions from Panel A with VDAC2 antibody. (B), Density gradient separation of the digitonin solubilized mouse mitochondrial tissue through sucrose density gradient centrifugation and visualized by Western blotting with a SCC antibody. Appearance of 57-kDa and 51-kDa SCC is indicated with black and red arrow. Bottom, The top membrane with longer exposure. (C), Western staining of the same digitonin lysate fractions with a VDAC2 antibody.

Figure S3 (Related to Figure 3). Difference in activity with pre and post incubation with succinate. (A), Thin layer chromatography showing the metabolic conversion pattern. (B), Analysis of the role of succinate due to pre and post incubation shown in a flow chart.

Figure S4 (Related to Figure 4). Metabolic activity determination. (A), Measurement of activity (^{14}C-cholesterol to pregnenolone conversion) with mitochondria isolated from MA-10 cells over different periods of time from 5 min to 2 hour. (B), Measurement of activity (^{3}H-pregnenolone to progesterone conversion) with mitochondria isolated from MA-10 cells as well as the digitonin lysate of the MA-10 mitochondria prepared in presence and absence of succinate. Trilostane (Tril) was added to inhibit 3βHSD2 activity to stop conversion after pregnenolone synthesis.

Figure S5 (Related to Figure 5). Expression analysis of the malate dehydrogenase. Top, Western blot analysis of malate dehydrogenase knock down stable clones 1 and 2, and its comparison with MA-10 cells with MDH2 antibody. Bottom, Western of the clones 1 and 2 from the top panel with pyruvate dehydrogenase (PDC-E2) antibody.

SUPPLEMENTAL REFERENCES

Bose, H.S., Gebrail, F., Marshall, B., Perry, E.W., and Whittal, R.W. (2019). Inner mitochondrial translocase Tim 50 is central in adrenal and testicular steroid synthesis. Mol Cell Biol *39*, doi:10.1128/MCB.00484-00418.

Bose, H.S., Lingappa, V.R., and Miller, W.L. (2002). Rapid regulation of steroidogenesis by mitochondrial protein import. Nature *417*, 87-91.

Bose, M., Whittal, R.M., Miller, W.L., and Bose, H.S. (2008). Steroidogenic activity of StAR requires contact with mitochondrial VDAC1 and phosphate carrier protein. J Biol Chem *283*, 8837-8845.

Marriott, K.C., Prasad, M., Thapliyal, V., and Bose, H.S. (2012). Sigma-1 receptor at the mitochondrial associated ER-membrane is responsible for mitochondrial metabolic regulation. J Pharmacol Exp Ther *343*, 578-586.

Pawlak, K.J., Prasad, M., McKenzie, K.A., Wiebe, J.P., Gairola, C.G., Whittal, R.M., and Bose, H.S. (2011a). Decreased cytochrome c oxidase IV expression reduces steroidogenesis. J Pharmacol Exp Ther *338*, 598-604.

Pawlak, K.J., Prasad, M., Thomas, J.L., Whittal, R.M., and Bose, H.S. (2011b). Inner mitochondrial translocase Tim50 interacts with 3beta-hydroxysteroid dehydrogenase type-2 to regulate adrenal and gonadal steroidogenesis. J Biol Chem *286*, 39130-39140

Prasad, M., Pawlak, K.J., Burak, W.E., Perry, E.E., Marshall, B., Whittal, R.M., and Bose, H.S. (2017). Mitochondrial metabolic regulation by GRP78. Science Advances *3*, e1602038.

Prasad, M., Thomas, J.L., Whittal, R.M., and Bose, H.S. (2012). Mitochondrial 3-beta hydroxysteroid dehydrogenase enzyme activity requires a reversible pH-dependent conformational change at the intermembrane space J Biol Chem *287*, 9534-9546.

Prasad, M., Walker, A.N., Kaur, J., Thomas, J.L., Powell, S.A., Pandey, A.V., Whittal, R.M., Burak, W.E., Petruzzelli, G., and Bose, H.S. (2016). Endoplasmic reticulum stress enhances

mitochondrial metabolic activity in mammalian adrenals and gonads. Mol Cell Biol *36*, 3058-3074.

Ragan, C.I., Wlson, M.T., Darley-Usmer, V.M., and Lowe, P.N. (1986). Sub-Fractionation of mitochondria and isolation of the proteins of oxidative phosphorylation (Washington, D.C.: IRL Press).

SUPPLEMENTAL FIGURES

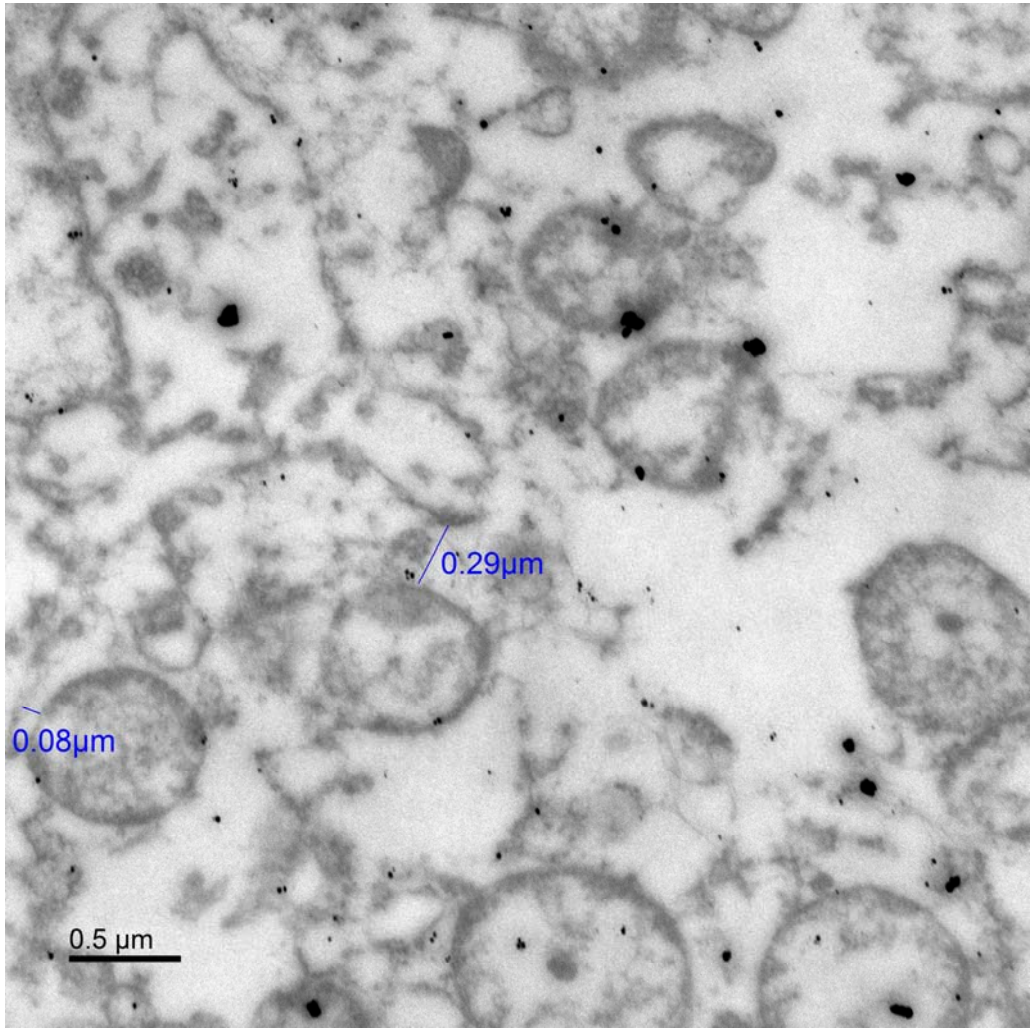

FIGURE S1

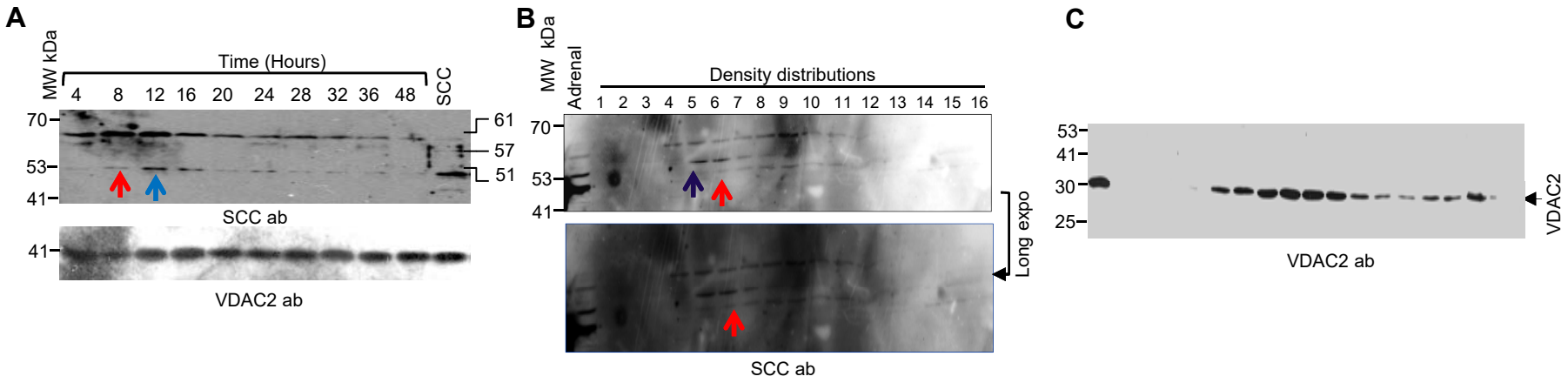
A
MW kDa
Time (Hours)
4 8 12 16 20 24 28 32 36 48 SCC
70
53
41
61
57
51
SCC ab
41
VDAC2 ab
B
MW kDa
Adrenal
Density distributions
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
70
53
41
Long expo
SCC ab
C
53
41
30
25
VDAC2
VDAC2 ab

FIGURE S2

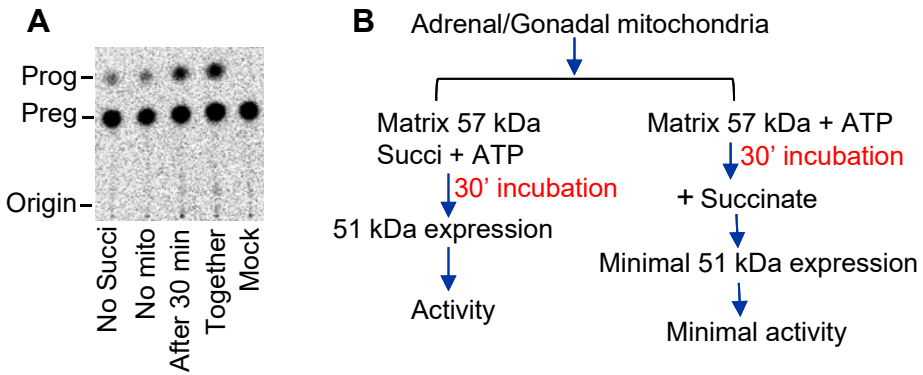
A
Prog
Preg
Origin
No Succi
No mito
After 30 min
Together
Mock
B
Adrenal/Gonadal mitochondria
Matrix 57 kDa
Succi + ATP
30' incubation
51 kDa expression
Activity
Matrix 57 kDa + ATP
30' incubation
+ Succinate
Minimal 51 kDa expression
Minimal activity

FIGURE S3

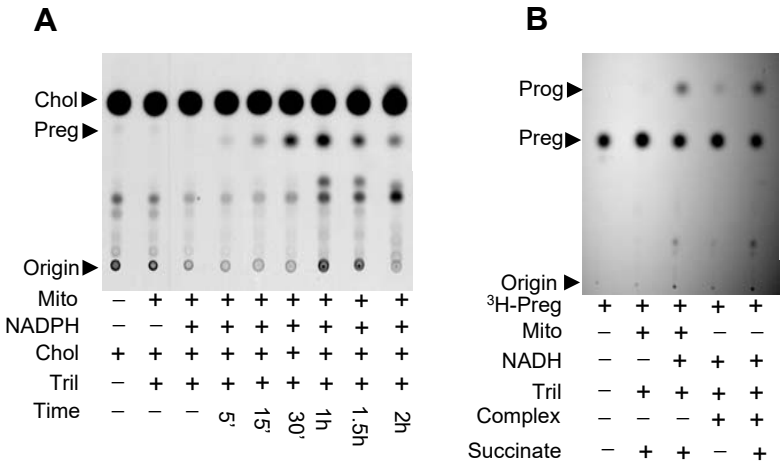
A
Chol
Preg
Origin
Mito – + + + + + + + +
NADPH – – + + + + + + +
Chol + + + + + + + + +
Tril – + + + + + + + +
Time – – – 5' 15' 30' 1h 1.5h 2h
B
Prog
Preg
Origin
3H-Preg + + + + +
Mito – + + – –
NADH – – + + +
Tril – + + + +
Complex – – – + +
Succinate – + + – +

FIGURE S4

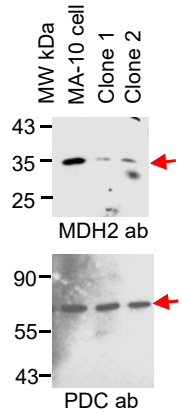

FIGURE S5