Supplemental Data:

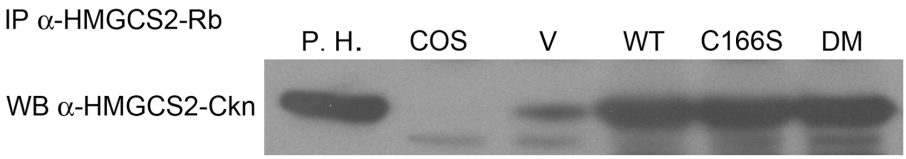

Figure S1 Expression of HMGCS2 in COS-7 cells transfected with wildtype and mutant HMGCS2 is similar to endogenous HMGCS2 from primary hepatocytes.

COS-7 cells transfected with 1 mg of pcDNA-HMGCS2-WT (WT), or pcDNA-HMGCS2-C166S (C166S) or pcDNA-HMGCS2-C166,305S (DM) and rat primary hepatocytes were immunoprecipitated with an anti-HMGCS from rabbit (a-HMGCS-Rb, our laboratory stock) antibody followed by SDS-PAGE and transfer to a PVDF membrane and probed with anti-HMGCS2 from ckicken (a-HMGCS2-Ckn, Abcam). Please note a portion of the WT sample likely leaked from the sample well and contaminated the vector lane (V), which is normally devoid on any signal as seen in figure 5B.

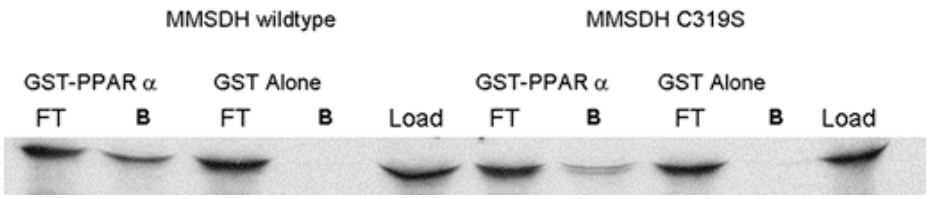

Figure S2 Comparison of the PPARa binding capabilities of wild-type MMSDH and palmitoylation deficient MMSDH C319S

A) *In vitro* transcribed and translated wildtype or palmitoylation deficient C319S mutant were incubated with 1 mg of GST/PPARa pre-bound to glutathione sepharose beads or GST sepharose alone as shown, followed by SDS-PAGE. The fluorogram of the dried gel representing 10 % of the flow through (FT) or the PPARa bound protein (B) is shown.

3-ketoacyl-CoA thiolase
Methylmalonate-semialdehyde dehydrogenase
Similar to Aldehyde dehydrogenase family 7 member A1
Enoyl-CoA hydratase
Electron transfer flavoprotein subunit alpha
Aspartate aminotransferase
Malate dehydrogenase
Heat shock protein 75 kDa
3-hydroxy-3-methylglutaryl-CoA synthase

Table S1. Palmitoylated mitochondrial proteins identified in Kostiuk et al. (26) and various *in situ* palmitoylated protein proteomics studies (31-33).

Protein name	SwissProt # or NCBI ID	MW (kDa)	NR box sequence	Residue #
Malate dehydrogenase	P04636	36	LSLLL	40-44
Methylmalonate-semialdehyde dehydrogenase	Q02253	58	LAKLL	221-225
Hydroxymethylglutaryl-CoA synthase	P22791	56	LASLL	390-394
Enoyl-CoA hydratase	P14604	32	LRALL	4-8
Dimethylglycine dehydrogenase	Q63342	96	LFPLL	165-169
Aldehyde dehydrogenase	P11884	57	LSRLL	15-19
Alanine--glyoxylate aminotransferase 2	Q64565	57	LSALL	151-155
Alpha-methylacyl-CoA racemase	P70473	42	LGILL	162-166

Table S2 Palmitoylated mitochondrial proteins containing LXXLL nuclear receptor binding motifs

Supplemental Data Materials and Methods

Plasmids

C-terminal hexa-His tagged versions of the mature cDNA for human mHMG-CoA synthase (HMGCS2-His_6), were constructed in the bacterial expression vector pET19b (Novagene) and have been described previously(26) pSG5PPARα and pGEX-GST/PPARα were a kind gift from Dr. John Capone, McMaster University, Canada. To produce the cysteine to serine mutants of HMGCS2-His_6, site directed mutagenesis was performed using the Stratagene Quickchange II site directed mutagenesis kit with the following primers:

Cys166Ser mutation

5' GAT ACC ACC AAT GCC T**C**C TAC GGT GGT ACT GCC

5' GGC AGT ACC ACC GTA G**G**A GGC ATT GGT GGT ATC

Cys305Ser mutation

5' C TTT CAT ACA CCC TTT T**C**C AAG ATG GTC CAG AAG TCT CTG G

5' C CAG AGA CTT CTG GAC CAT CTT G**G**A AAA GGG TGT ATG AAA G

Cys166,305Ser double mutation the pET19b- HMGCS-His_6-C166S vector was subjected to site directed mutagenesis with the above primers for C305S mutation.

The bases that were exchanged resulting in the substitution of the original codon encoding for a cysteine residue to a codon encoding for a serine residue are underlined.

All vectors were sequenced by MWG Biotech Inc. (Huntsville, AL 35805 USA) prior to protein expression. The HA tagged pCDNA-HMGCS2-HA wildtype and mutant plasmids for expression in COS-7 cells were engineered by PCR using the HMGCS2 coding region from the pET19b plasmids as templates with the following primers:

Forward 5'AAGCTTCGAATTCATG CAG CGT CTG TTG ACT CCA GTG AAG CGC

Reverse 5' ACTTCGGATCCTTA AGC GTA ATC TGG AAC ATC GTA TGG GTA GAC GGG ACG CCG GGC ATA CTT TCG

Amplified PCR products were cut with BamH1 and EcoR1 and sub cloned in appropriately digested pCDNA 3.1- plasmid.

Mass spectrometry analysis of HMGCS2WTand mutants.

Chemicals

Ammonium bicarbonate (NH_4HCO_3), Iodoacetamide (for carbamidomethylation of free thiols), dithiothreitol (DTT), N-ethylmaleimide (NEM), alpha-cyano-4-hydroxycinnamic acid (4-HCCA), universal matrix (1:1 mixture of 4-HCCA and 2,5 dihydroxybenzoic acid (DHB)) and trifluoroacetic acid were purchased from Sigma-Aldrich Canada (Oakville, ON) and used without further purification. Deuterated NEM (NEM-d5) was purchased from Cambridge Isotopes (MA, USA). Solvents were from Fisher Scientific Canada (Milton, ON) and of HPLC grade.

Reduction, alkylation and digestion of proteins

Approximately 5 µg of WT HMGCS-His_6 and mutants (palmitoylated or non-palmitoylated) in a volume of 5 µl buffer was reduced with 2.5 µl 80 mM DTT and kept at 37°C for 30 min. This step was followed with adding a 2.5 µl volume of alkylating reagent (iodoacetamide, NEM or NEM-d_5) at a concentration of 200 mM. After addition of the alkylation reagent the samples were kept at room temperature (RT) for 1 hour in the dark. Subsequently the pH was elevated with addition of 1 µl of 1M NH_4HCO_3 and 0.5 µl of a 2µg/µl enzyme solution in dd. H_2O was added. The sample was then incubated at RT overnight.

Mass spectrometric analysis of tryptic and chymotryptic peptides

For peptide mapping and fragmentation analysis with matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI TOF MS/MS), each peptide digestion mixture was purified with a μC_{18}-Ziptip (Millipore, Billerica, MA) pipette tip. The bound peptides in the ZipTip were eluted in gradient steps using 4 µl of each 25%, 40%, 65% and 85% ACN in 0.1% TFA. Gradient fractions were then mixed 1:1 with following matrix solution: 20 mg/ml universal matrix in 1:1 ACN:H_2O. For sample spotting a modified two-layer method was employed as described by Dai et al. (44). Briefly, ~1µl of a 12 mg/ml 4-HCCA solution of 80:20 v/v acetone:methanol was spotted onto the MALDI stainless steel target to form a thin first layer. As a second layer 0.5 µl of the 1:1 peptide extract matrix mixture was spotted onto the dried first layer and air dried. The MALDI targets were analyzed with a Voyager DE-STR MALDI TOF MS (Applied Biosystems, Framingham, MA), located at the UBC Proteome Core Facility. The instrument was operated in positive reflectron mode. Calibration was done first externally with known peptide standards, before data analysis. Internal calibration was performed with known matrix cluster signals and tryptic autolysis peptide signals, to achieve a mass accuracy of less than 100 ppm. About 300-500 single laser shot spectra were accumulated per sample. Further confirmation of the peptide identities was performed by MALDI-TOF/TOF fragmentation analysis of selected tryptic or chymotryptic peptides as indicated in the text. The instrument used was a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA) located at the UBC Biomedical Research Centre. The instrument was also operated in positive reflectron mode. Again, calibration was done first externally with known peptide standards, before data analysis.

Immunoprecipitation

Post-nuclear supernatants from COS-7 cells transfected with 1μg pcDNA-HMGCS2-HA wild-type or 1μg pcDNA-HMGCS2-HA-Cys166Ser or 1μg pcDNA-HMGCS2-HA-Cys166,305Ser and primary hepatocytes lysed with RIPA 1% SDS buffer were incubated with 5 μg affinity purified rabbit anti-HMGCS2 from our laboratory for 2 hours at room temperature with rocking followed by incubation with 20 μl protein G sepharose. Following extensive washes the beads were then boiled 2 minutes in 1x SDS loading buffer containing 50 mM DTT and subjected to SDS-PAGE and Western blotting with chicken anti-HMGCS2 (Sigma-Aldrich) and ECL detection.

References

Dai, Y., Whittal, R.M., and Li, L. (1999) Two-layer sample preparation: a method for MALDI-MS analysis of complex peptide and protein mixtures. *Analytical chemistry* **71:** 1087-1091.

Kostiuk, M.A., Corvi, M.M., Keller, B.O., Plummer, G., Prescher, J.A., Hangauer, M.J., Bertozzi, C.R., Rajaiah, G., Falck, J.R., and Berthiaume, L.G. (2008) Identification of palmitoylated mitochondrial proteins using a bio-orthogonal azido-palmitate analogue. *FASEB J* **22:** 721-732.