Phosphorylation of Ser-204 and Tyr-405 in human malonyl-CoA decarboxylase expressed in silkworm *Bombyx mori* regulates catalytic decarboxylase activity

著者	Hwang In-Wook, Makishima Yu, Suzuki Tomohiro, Kato Tatsuya, Park Sungjo, Terzic Andre, Chung Shin-kyo, Park Enoch Y.
journal or publication title	Applied Microbiology and Biotechnology
volume	99
number	21
page range	8977-8986
year	2015-05-26
出版者	Springer
権利	(C) Springer-Verlag Berlin Heidelberg 2015. The final publication is available at Springer via https://doi.org/10.1007/s00253-015-6687-x
URL	http://hdl.handle.net/10297/9422

doi: 10.1007/s00253-015-6687-x

Phosphorylation of Ser-204 and Tyr-405 in human malonyl-CoA decarboxylase expressed in silkworm *Bombyx mori* regulates catalytic decarboxylase activity

In-Wook Hwang · Yu Makishima · Tomohiro Suzuki · Tatsuya Kato · Sungjo Park · Andre Terzic · Shin-kyo Chung · Enoch Y. Park

In-Wook Hwang · Enoch Y. Park (✉)
Laboratory of Biotechnology, Integrated Bioscience Section, Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka, 422-8529, Japan. E-mail: park.enoch@shizuoka.ac.jp; Tel. & Fax: +81-54-2384887

Yu Makishima · Tatsuya Kato · Enoch Y. Park
Laboratory of Biotechnology, Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka, 422-8529, Japan.

Tomohiro Suzuki
Research Institute of Green Science and Technology, Shizuoka University, 836 Ohya Suruga-ku, Shizuoka, 422-8529, Japan.

Tatsuya Kato · Enoch Y. Park
Laboratory of Biotechnology, Green Chemistry Research Division, Research Institute of Green Science and Technology, Shizuoka University, 836 Ohya Suruga-ku, Shizuoka, 422-8529, Japan.

Sungjo Park · Andre Terzic
Center for Regenerative Medicine, Mayo Clinic, 200 First Street SW, Rochester, MN, 55905, USA. Marriott Heart Disease Research Program, Division of Cardiovascular Diseases, Departments of Medicine, Molecular Pharmacology and Experimental Therapeutics, and Medical Genetics, Mayo Clinic, 200 First Street SW, Rochester, MN, 55905, USA.

Shin-kyo Chung
School of Food Science and Biotechnology, Kyungpook National University, Daegu 702-701, Republic of Korea

Authors' E-mail address;

In-Wook Hwang: gunryung21@hanmail.net (IH)

Yu Makishima: mmm.yu@live.jp (YM)

Tomohiro Suzuki: atsuzuk@ipc.shizuoka.ac.jp (TS)

Tatsuya Kato: atkato@ipc.shizuoka.ac.jp (TK)

Sungjo Park: park.sungjo@mayo.edu (SP)

Andre Terzic: terzic.andre@mayo.edu (AT)

Shin-kyo Chung: kchung@knu.ac.kr (SC)

Enoch Y. Park: park.enoch@shizuoka.ac.jp (EYP)

ABSTRACT

Decarboxylation of malonyl-CoA to acetyl-CoA by malonyl-CoA decarboxylase (MCD; EC 4.1.1.9) is a vital catalytic reaction of lipid metabolism. While it is established that phosphorylation of MCD modulates the enzymatic activity, the specific phosphorylation sites associated with the catalytic function have not been documented due to lack of sufficient production of MCD with proper post-translational modifications. Here, we used the silkworm-based BmNPV bacmid system to express human MCD (hMCD) and mapped phosphorylation effects on enzymatic function. Purified MCD from silkworm displayed post-translational phosphorylation and demonstrated coherent enzymatic activity with high yield (~200 µg/silkworm). Point mutations in putative phosphorylation sites, Ser-204 or Tyr-405 of hMCD, identified by bioinformatics and proteomics analyses reduced the catalytic activity, underscoring the functional significance of phosphorylation in modulating decarboxylase-based catalysis. Identified phosphorylated residues are distinct from the decarboxylation catalytic site, implicating a phosphorylation-induced global conformational change of MCD as responsible in altering catalytic function. We conclude that phosphorylation of Ser-204 and Tyr-405 regulates the decarboxylase function of hMCD leveraging the silkworm-based BmNPV bacmid expression system that offers a fail-safe eukaryotic production platform implementing proper post-translational modification such as phosphorylation.

Keywords Human malonyl-CoA decarboxylase (hMCD) · Site directed mutagenesis · Phosphorylation/dephosphorylation · Lipid metabolism · Silkworm · *Bombyx mori* nucleopolyhedrovirus

62

63 **Introduction**

64 Malonyl-CoA decarboxylase (MCD, E.C.4.1.1.9), encoded by *MLYCD*, is the crucial
65 metabolic enzyme responsible for sustaining homeostatic lipid metabolism (Dyck et al. 2006;
66 Folmes et al. 2013; Saggerson 2008). Due to the importance of cellular functions in fatty acid
67 metabolism, MCD is ubiquitously expressed in all living organisms locating in mitochondria,
68 peroxisome and cytoplasm (Buckner et al. 1976; Kim et al. 1979; Scholte 1969). In humans,
69 deficiency of MCD (OMIM 248360) precipitates a spectrum of disorders including
70 cardiomyopathy, hypoglycaemia, hypotonia, mild mental retardation, metabolic acidosis,
71 malonic aciduria, seizures and vomiting (Brown et al. 1984; Haan et al. 1986; Krawinkel et al.
72 1994; MacPhee et al. 1993; Matalon et al. 1993; Xue et al. 2012; Yano et al. 1997).

73 MCD participates in the degradation of malonyl-CoA, an integral metabolic intermediate
74 in anabolic/catabolic lipid metabolism (Figures S1 and S2, Supporting Information).
75 Malonyl-CoA is a committed substrate for *de novo* fatty acid biosynthesis, yet abundant
76 malonyl-CoA inhibits carnitine palmitoyltransferase 1 (CPT-1), a rate-limiting step for long-
77 chain fatty acid transport into mitochondria and subsequent lipid β -oxidation (Kim et al.
78 1989; Pender et al. 2006). Inhibition of MCD reduces fatty acid β -oxidation and accelerates
79 glucose oxidation, producing a metabolic switch in energy substrate preference (Dyck et al.
80 2006). Consequently, regulation of MCD activity to modulate intracellular malonyl-CoA
81 levels is considered for potential therapeutic applications to mitigate metabolic disorders.

82 Post-translational modification of MCD modulates enzymatic function (Dyck et al. 2000;
83 Laurent et al. 2013; Park et al. 2002; Saha et al. 2000; Sambandam et al. 2004; Voilley et al.
84 1999). For example, MCD has several acetylation sites and MCD deacetylation inhibits the
85 decarboxylase activity thereby promoting *de novo* lipogenesis, whereas MCD acetylation

enhances fatty acid oxidation (Laurent et al. 2013). In contrast, the catalytic activity associated with MCD phosphorylation/dephosphorylation has not been definitively documented as phosphorylation sites have not been identified and production of sufficient authentic MCD with suitable post-translational modification remains limited (Dyck et al. 2000; Park et al. 2002; Saha et al. 2000; Sambandam et al. 2004; Voilley et al. 1999).

The *E. coli* expression system has been widely used for recombinant protein production, yet the proteins expressed in this system often show poor post-translational modifications (Kamionka 2011). To secure genuine eukaryotic protein production with proper post-translational modifications, a *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid, a hybrid shuttle vector for an *E. coli* and *B. mori*, has been developed (Kato et al. 2010; Motohashi et al. 2005). With this system, we have successfully produced cellular, mitochondrial, and membrane proteins with proper folding and post-translational modifications (Figure S3, Supporting Information) (Dojima et al. 2009; Du et al. 2009; Hwang et al. 2014).

Here, we examined whether phosphorylation of human MCD (hMCD) generated using the silkworm-based BmNPV bacmid expression system modulates the essential decarboxylase activity. We report that the recombinant hMCD displays phosphorylation properties with consistent catalytic activity. Point mutations in the phosphorylation sites, namely Ser-204 and Tyr-405, limit the enzymatic activity of hMCD, underscoring the regulation of catalytic function by phosphorylation. The silkworm-based BmNPV bacmid expression system thus provides a reliable recombinant eukaryotic protein production modality with proper post-translational phosphorylation suitable for functional analysis of human MCD.

Materials and Methods

Construction of recombinant $\Delta 39$ aa-hMCD BmNPV bacmid and mutants

The overall strategy for the construction and expression of $\Delta 39\text{aa-hMCD}$ BmNPV bacmid is shown in Figure 1. The complementary DNA of hMCD from Mammalian Gene Collection (GenBank EAW95513.1, Thermo Scientific, Pittsburgh, PA, USA) was used as a template. N-terminal 39 amino acids of hMCD, a putative mitochondria targeting sequence, were deleted using conventional polymerase chain reaction (PCR) with a pair of primers containing the *Bam*HI/*Xho*I restriction cloning site: 5'-GCGGATCCCACCATGGACTACAAGGATGACGATGACAAGATGGACGAGCTGCTGC GCCGC-3' (forward), 5'-GCCTCGAGTCAGAGCTTGCTGTTCTTTTGAAACTG-3' (reverse). Deletion of the mitochondria targeting sequence leads to high protein expression and does not affect the enzyme activity (Zhou et al. 2004). In addition, the Kozak consensus sequence and the FLAG tag sequence were attached at N-terminus for high expression levels in baculovirus expression system and for purification of expressed protein, respectively. The PCR cycle was conducted following 40 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 5 s, and extension at 72°C for 10 s using PrimeSTAR[®] Max premix kit (Takara Bio Inc., Otsu, Shiga, Japan). The resultant PCR product ($\Delta 39\text{aa-hMCD}$ gene) was digested with *Bam*HI and *Xho*I followed by purification with a GFX[™] PCR DNA and Gel Band Purification Kit (GE Healthcare, Amersham, UK). The purified DNA fragment was ligated into pFastbac 1 vector, transformed into *E. coli* competent DH5 α cells (Invitrogen, Carlsbad, CA, USA) and then cultured on a solid LB medium containing 100 $\mu\text{g/mL}$ of ampicillin at 37°C for 18 h. The plasmid containing $\Delta 39\text{aa-hMCD}$ gene was isolated and its sequence identity was confirmed by DNA sequencing. Finally, *E. coli* BmDH10bac-CP⁻-Chi⁻ competent cells containing the cysteine proteinase- and chitinase-deficient BmNPV bacmid (Park et al. 2008a) were transformed with the pFastbac1- $\Delta 39\text{aa-hMCD}$ and cultured on a solid LB medium containing 50 $\mu\text{g/mL}$ of kanamycin, 7 $\mu\text{g/mL}$ of gentamycin, 10 $\mu\text{g/mL}$ of tetracycline, 40 $\mu\text{g/mL}$ of isopropyl β -D-1-thiogalactopyranoside (IPTG) and 100 $\mu\text{g/mL}$ of

5-bromo-4-chloro-3-indolyl-4-galactoside (X-Gal) (Takara Bio) at 37°C for 18 h (Figure S4, Supporting Information). To confirm the BmNPV-Δ39aa-hMCD, bacmid PCR of white colonies was assessed using M13 primers. The PCR cycle was conducted following 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 10 s, and extension at 72°C for 40 s using SapphireAmp Fast PCR Master Mix kit (Takara Bio). The recombinant BmNPV bacmid (BmNPV-Δ39aa-hMCD) was isolated from positive colonies confirmed by bacmid PCR.

For the construction of mutated hMCD BmNPV bacmid (S204G and Y405F), the QuikChangeII XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used according to the manufacturer's protocols. The pFastbac-Δ39aa-hMCDs containing the desired mutation were produced using the constructed pFastbac-Δ39aa-hMCD as a template by PCR reaction with primer sets containing changed base for mutation: 5'-GGGTTACCTGGCATGGACCGTGTGAAGTGCTTC-3' (forward primer for S204G), 5'-GAAGCACTTCACACGGTCCATGCCAGGTAACCC-3' (reverse primer for S204G), 5'-AGGCTGTGCGCCTGGTTCCTGTATGGAGAGAAG-3' (forward primer for Y405F), 5'-CTTCTCTCCATACAGGAACCAGGCGCACAGCCT-3' (reverse primer for Y405F). The PCR cycle was conducted following 18 cycles of denaturation at 95°C for 15s, annealing at 60°C for 30s, and extension at 68°C for 6.5 min. After *Dpn* I treatment at 37°C for 3 hr, *Dpn* I-treated plasmids were transformed into XL10-Gold ultracompetent cells (Stratagene). The plasmid containing pFastbac-Δ39aa-hMCD mutant genes were isolated and its sequence identity with mutation was confirmed by DNA sequencing.

Expression and purification of recombinant Δ39aa-hMCD in silkworm larvae and pupae

To produce a recombinant protein in silkworm, 10 μg of each recombinant BmNPV bacmid DNA was directly injected with DMRIE-C reagent (Invitrogen) into the dorsum of larvae and

pupae using a syringe with a 26-gauge beveled needle (Terumo Co. Tokyo, Japan). The injected larvae and pupae were reared at 27°C in an incubator for 6–7 days. In the case of larvae, the fat body was collected by cutting and dissection. The samples including fat body or pupae were immediately frozen at –80°C until further analysis. Protein purification was carried out at 4°C to minimize aggregation and protease activity. The aliquot of collected larval fat body or pupae was homogenized in 10 mL of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4 and 0.1% TritonX-100) containing an EDTA-free protease inhibitor tablet (Roche, Mannheim, Germany) using a homogenizer (GLH-115, Yamato, Tokyo, Japan). Cell debris was removed by pelleting through centrifugation at 12,000 *g* for 30 min. The supernatant was filtered using a 0.45 µm syringe filter and loaded onto a 500 µL of anti-FLAG M2 antibody affinity gel (Sigma-Aldrich, St. Louis, MO, USA) pre-equilibrated with equilibration buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4 and 0.02% TritonX-100). The column was washed with 2.5 mL of equilibration buffer and eluted with elution buffer (100 µg/mL FLAG peptide in 50 mM Tris-HCl and 150 mM NaCl, pH 7.4). Purified protein concentration was determined using BCA protein assay kit (Thermo Scientific, Rockford, IL, USA).

SDS-PAGE and Western blotting

The integrity of purified Δ39aa-hMCD was determined by Coomassie brilliant blue (CBB)-staining and western blotting analyses (Karger et al. 2008; Park et al. 2008b). Prior to electrophoresis, purified samples were boiled for 5 min at 95°C with protein denaturing buffer (Nacalai Tesque, Kyoto, Japan). Samples were electrophoresed in a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel with a Mini-protean system (Bio-Rad, Hercules, CA, USA) at 150 V for 45–60 min in Tris-glycine buffer (25 mM Tris, 250 mM glycine, pH 8.3 and 0.1% SDS). After electrophoresis, the SDS-PAGE gel was

stained with Coomassie blue staining solution. For Western blotting analysis, the separated proteins on a SDS-PAGE gel were transferred to PVDF membranes (GE Healthcare) by electroblotting on a semi-dry blotter (Bio-Rad) at 15 V for 1 h. To detect the purified $\Delta 39aa$ -hMCD and their phosphorylation, a mouse anti-FLAG antibody (Wako Pure Chem. Ind. Ltd., Osaka, Japan), anti-phosphoserine antibody and anti-phosphotyrosine (Sigma-Aldrich) were used as primary antibodies, respectively. An anti-mouse IgG-HRP (GE Healthcare) was used as a secondary antibody in both cases.

MCD activity assay

The hMCD activity was measured by the production of NADH in the coupled reactions (Kim and Kolattukudy 1978). The NADH is accumulated by the reaction of acetyl-CoA from malonyl-CoA and oxaloacetate from malate (Eqs. 1–3).

To measure the decarboxylase activity of recombinant hMCD, 10 μg of purified $\Delta 39aa$ -hMCD was incubated with 36 μL of reaction buffer (10 mM of Tris-HCl, pH 7.5, 2 mM of malate, 2 mM of NAD, 0.8 U of malate dehydrogenase) at room temperature. After 5 min, assay was initiated by the addition of 3 mM of malonyl-CoA and incubated at 37°C. After 10 min, 0.03 U of citrate synthase was added to the incubated solution. The reaction was terminated by addition of 4 μL of 100% trichloroacetic acid. The produced NADH during reaction was detected using an UV-visible spectrophotometer by measuring the absorbance at 340 nm.

Results

Expression and purification of $\Delta 39\text{aa-hMCD}$

The hMCD harbors a mitochondrial targeting sequence (39 amino acids) at N-terminus and a peroxisomal targeting sequence (Ser-Lys-Leu, SKL) at C-terminus (Fig. 1). The peroxisomal SKL residues do not affect the protein expression levels, but the mitochondrial targeting sequence significantly diminishes MCD expression (Voilley et al. 1999). Therefore, we designed a construct without a mitochondrial targeting sequence but retaining the peroxisomal SKL sequence to enhance the recombinant hMCD production (Fig. 1). The recombinant $\Delta 39\text{aa-hMCD}$ with an N-terminal FLAG tag was expressed in silkworm larvae and pupae, and purified using an anti-FLAG M2 affinity gel column. The purified $\Delta 39\text{aa-hMCD}$ protein with more than 95% purity migrated to ~50 kDa, an estimated molecular weight on SDS-PAGE based on comparison with molecular weight markers (Fig. 2A). Western blot analysis using a FLAG-specific antibody confirmed the specific expression of $\Delta 39\text{aa-hMCD}$ (Fig. 2B). In mock-injected silkworm, however, hMCD bands were not detected in both fat body and pupae samples (Fig. 2B). Thus, the recombinant $\Delta 39\text{aa-hMCD}$ purified here demonstrated significant homogeneity suitable for biochemical and functional analyses.

Silkworm-based recombinant protein expression conventionally provides a high yield of purified proteins in the range of 20 – 500 $\mu\text{g/silkworm}$ (Table 1). Consistent with this, the average yields of purified $\Delta 39\text{aa-hMCD}$ from ten silkworm larvae and five pupae were 119 $\mu\text{g/larva}$ and 344 $\mu\text{g/pupa}$. The pupae-based protein expression provided a higher yield of purified protein than the fat body-based expression, probably due to the difference of organ size and protease activity in the expression host. Specific decarboxylase activities of the purified $\Delta 39\text{aa-hMCD}$, measured by the production of NADH in the coupled reactions, were

59.54 $\pm$ 7.68 ($n = 6$) nmol/mg/min from silkworm fat body and 48.16 $\pm$ 7.89 ($n = 6$) nmol/mg/min from silkworm pupae (Table 2). These measured enzymatic activities showed no significant statistical difference ($p>0.05$), but are much higher than that of human recombinant MCD purified from *E. coli* (Zhou et al. 2004) indicating that the post-translational modifications implemented in silkworms regulate catalytic activity.

Phosphorylation-induced catalytic function of $\Delta 39$ aa-hMCD

The catalytic activity of MCD associated with phosphorylation/dephosphorylation was previously evaluated (Dyck et al. 1998; Park et al. 2002; Saha et al. 2000), yet these studies provided inconsistent results either a decrease or increase in decarboxylase activity of MCD. To clarify the phosphorylation-induced effects on MCD enzymatic function, potential phosphorylation sites of hMCD were examined. NetPhos 2.0, neural network predictions for serine, threonine and tyrosine phosphorylation in eukaryotic proteins (<http://www.cbs.dtu.dk/services/NetPhos/>), was applied to predict hMCD phosphorylation sites (Blom et al. 1999) (Fig. 3A). Four serine (Ser-204, Ser-275, Ser-326, Ser-380), four threonine (Thr-9, Thr-60, Thr-245, Thr-396) and one tyrosine (Tyr-468) residues were putatively identified as phosphorylation sites, which are well conserved (Fig. 3A & 3B). Typically, each phosphorylation prediction program maps slightly different potential amino acid residues depending on bioinformatics algorithm. Based on the NetPhos 2.0 prediction with 9 amino acid residues as potential phosphorylation sites, we performed the mass spectrometry analysis to narrow down the proteomic search, and identified exclusive phosphorylation of Ser-204 and Tyr-405 in the recombinant $\Delta 39$ aa-hMCD, thereby excluding the investigation of other predicted phosphorylation sites. Accordingly, these residues were mutated to glycine (S204G) and phenylalanine (Y405F) using site-directed mutagenesis to eliminate the hydroxyl group for phosphorylation (Fig. 3C).

The point mutants S204G and Y405F were purified from silkworm fat body and pupae, and analyzed by Western blotting using an anti-phosphoserine and anti-phosphotyrosine antibodies (Fig. 4). The $\Delta 39\text{aa}$ -hMCD mutants displayed a substantial decrease on Western blotting analysis compared with the wild type $\Delta 39\text{aa}$ -hMCD, indicating that Ser-204 and Tyr-405 are indeed the residues for phosphorylation. The lack of complete absence of the phosphorylated MCD band in Western blotting, however, could be ascribed to phosphorylated amino acid residues other than Ser-204 and Tyr-405.

To evaluate the effect of phosphorylation on the biological function of hMCD, the decarboxylase activity of $\Delta 39\text{aa}$ -hMCD mutants were measured. The specific activities of S204G mutant purified from fat body and pupae were 30.36 ± 2.25 ($n = 6$) and 24.37 ± 1.99 nmol/mg/min ($n = 6$), respectively, which are lower by 50% than that of wild type $\Delta 39\text{aa}$ -hMCD (Table 3). In addition, the specific activities of Y405F mutants were 33.45 ± 3.56 ($n = 6$) and 31.24 ± 1.69 nmol/mg/min ($n = 6$) purified from fat body and pupae, respectively. Collectively, the dephosphorylation of hMCD diminishes decarboxylase activity, underlining the phosphorylation-induced regulation in catalytic function.

Structural implication of hMCD phosphorylation

The resolved crystal structure of hMCD (PDB accession number: 4F0X) reveals a molecular tetramer, composed by a dimer of structural heterodimer where the two subunits show different conformations (Fig. 5) (Aparicio et al. 2013). The monomer of MCD has an N-terminal helical domain for oligomerization and a C-terminal domain for catalysis, and the active site of MCD is located in a prominent groove clustered with evolutionarily conserved residues (Fig. 5A) (Aparicio et al. 2013; Froese et al. 2013). Through inter-subunit disulfide bonds, Cys-206—Cys-206 and Cys-243—Cys-243 (Fig. 5B), the four subunits of the tetramer are connected, providing positive cooperativity to the decarboxylase catalytic

function (Aparicio et al. 2013).

Ser-204 identified here for the phosphorylated residue is located in the beginning of the catalytic domain. The side chain of Ser-204 is fully exposed to solvent in the monomeric structure (Fig. 5A & 5C), and ~20 Å away from the active site, suggesting that the phosphorylation could not directly affect the structural integrity of the catalytic active site. However, the quaternary structure of hMCD reveals that Ser-204 is located nearby the Cys-206, the essential residue for the inter-subunit disulfide bond interaction (Fig. 5C). Thus, the phosphorylation/dephosphorylation of Ser-204 might modulate the disulfide bridge formation, contributing to the catalytic function. Moreover, as Lys-210 (rat sequence; Lys-211 in human) has been reported to be an essential amino acid residue for rat MCD enzymatic function through acetylation (Nam et al. 2006), the post-translational modifications in the vicinity of Cys-206 could be a key player in regulation of MCD function.

In contrast to Ser-204, Tyr-405 is not in the vicinity of inter-subunit interactions. This residue is located near to the catalytic dyad with His-423 and Ser-329. The hydroxyl group of Tyr-405 forms a hydrogen bond with the side chain of Asn-417, which is in the same α -helix harboring catalytic His-423 (Fig. 5D). Based on this structural information, the phosphorylation of Tyr-405 might induce the conformational change of a catalytic dyad producing the fine-tuning in decarboxylase activity.

Discussion

Malonyl-CoA decarboxylase is a metabolic enzyme participating in the production of acetyl-CoA from malonyl-CoA, a vital metabolite for anabolic fatty acid biosynthesis and catabolic lipid oxidation (Dyck et al. 1998; Pender et al. 2006). MCD overexpression increases fatty acid oxidation and improves whole body insulin resistance (An et al. 2004), whereas reduced MCD levels decrease lipid oxidation with an increase in glucose oxidation in human

myotubes (Bouzakri et al. 2008). In addition, catalytic function of MCD is regulated by multiple factors including post-translational modifications (Dyck et al. 2000; Nam et al. 2006; Park et al. 2002; Saha et al. 2000). Due to the pathophysiological relevance of MCD in metabolism-associated disorders, the regulation of MCD activity has been increasingly recognized as a candidate target for therapeutic interventions, essentially requiring authentic heterologous protein production to map critical molecular entities (Sambandam et al. 2004; Saggerson 2008). Here, we successfully produced recombinant hMCD using the silkworm-based BmNPV expression system equipped with a proper post-translational modification machinery. The heterologous MCD from silkworm demonstrated an essential and higher decarboxylase activity compared to that of MCD from *E. coli* (Zhou et al. 2004), underscoring the enzymatic activity enhancement by post-translational modifications implemented in silkworm. Furthermore, we identified two critical phosphorylated residues of Ser204 and Tyr-405 involved in regulation of decarboxylase-based catalysis.

AMP-activated protein kinase (AMPK) has been suggested to regulate MCD activity. Phosphorylation of MCD by AMPK promotes the enzymatic activity, leading to a decrease in the malonyl-CoA levels, whereas dephosphorylation inhibits the catabolic function (Park et al. 2002; Saha et al. 2000). Up to date, several putative phosphorylation sites on serine, threonine and tyrosine residues have been suggested, (Voilley et al. 1999), yet critical phosphorylation residues affecting the human MCD activity have remained elusive. By utilizing bioinformatics and mass spectrometry analyses, we identified exclusive phosphorylation of Ser-204 and Tyr-405. Point mutations of the phosphorylation sites decreased specific activity of hMCD, highlighting the biological significance of Ser-204 and Tyr-405 in regulating decarboxylase activity.

In summary, silkworm-based BmNPV protein expression system successfully produced the human MCD with high yield and post-translation modification. Heterologous MCD

retains essential decarboxylase activity and harbors two post-translational phosphorylation residues, namely Ser-204 and Tyr-405, critical in modulating MCD catalytic function. Identified phosphorylated residues are distinct from the decarboxylation catalytic site, implicating a phosphorylation-induced global conformational change of MCD as responsible in altering catalytic function. Collectively, our findings demonstrate that phosphorylation modulates decarboxylase-based catalytic function of MCD leveraging the silkworm-based BmNPV expression system that offers a fail-safe eukaryotic bioengineered protein production platform implementing phosphorylation. Furthermore, authentic recombinant proteins produced from silkworm could be used for functional and structural studies including high-throughput therapeutic drug discovery application.

Note

The authors declare that they have no competing interests.

Acknowledgements

This work was supported partly by the Strategic Young Researcher Overseas Visits Program for Accelerating Brain Circulation from Japan Society for the Promotion of Science (JSPS), Japan, the National Institute of Health, Marriott Heart Disease Research Program, Mayo Clinic. A.T. holds the Marriott Family Professorship in Cardiovascular Diseases Research.

References

An J, Muoio DM, Shiota M, Fujimoto Y, Cline GW, Shulman GI, Koves TR, Stevens R, Millington D, Newgard CB (2004) Hepatic expression of malonyl-CoA decarboxylase reverses muscle, liver and whole-animal insulin resistance. *Nat Med* 10:268–74. doi: 10.1038/nm995

348 Aparicio D, Pérez-Luque R, Carpena X, Díaz M, Ferrer JC, Loewen PC, Fita I (2013)
 349 Structural asymmetry and disulfide bridges among subunits modulate the activity of
 350 human malonyl-CoA decarboxylase. J Biol Chem 288:11907–19. doi:
 351 10.1074/jbc.M112.443846

352 Blom N, Gammeltoft S, Brunak S (1999) Sequence and structure-based prediction of
 353 eukaryotic protein phosphorylation sites. J Mol Biol 294:1351–62. doi:
 354 10.1006/jmbi.1999.3310

355 Bouzakri K, Austin R, Rune A, Lassman ME, Garcia-Roves PM, Berger JP, Krook A,
 356 Chibalin A V, Zhang BB, Zierath JR (2008) Malonyl CoenzymeA decarboxylase
 357 regulates lipid and glucose metabolism in human skeletal muscle. Diabetes 57:1508–16.
 358 doi: 10.2337/db07-0583

359 Brown GK, Scholem RD, Bankier A, Danks DM (1984) Malonyl coenzyme A decarboxylase
 360 deficiency. J Inherit Metab Dis 7:21–6.

361 Buckner JS, Kolattukudy PE, Poulouse AJ (1976) Purification and properties of malonyl-
 362 coenzyme A decarboxylase, a regulatory enzyme from the uropygial gland of goose.
 363 Arch Biochem Biophys 177:539–51.

364 Dojima T, Nishina T, Kato T, Uno T, Yagi H, Kato K, Park EY (2009) Comparison of the N-
 365 linked glycosylation of human beta1,3-N-acetylglucosaminyltransferase 2 expressed in
 366 insect cells and silkworm larvae. J Biotechnol 143:27–33. doi:
 367 10.1016/j.jbiotec.2009.06.013

368 Du D, Kato T, Suzuki F, Park EY (2009) Expression of protein complex comprising the
 369 human prorenin and (pro)renin receptor in silkworm larvae using *Bombyx mori*

370 nucleopolyhedrovirus (BmNPV) bacmids for improving biological function. *Mol*
 371 *Biotechnol* 43:154–61. doi: 10.1007/s12033-009-9183-7

372 Dyck JR, Barr AJ, Barr RL, Kolattukudy PE, Lopaschuk GD (1998) Characterization of
 373 cardiac malonyl-CoA decarboxylase and its putative role in regulating fatty acid
 374 oxidation. *Am J Physiol* 275:H2122–9.

375 Dyck JR, Berthiaume LG, Thomas PD, Kantor PF, Barr AJ, Barr R, Singh D, Hopkins TA,
 376 Voilley N, Prentki M, Lopaschuk GD (2000) Characterization of rat liver malonyl-CoA
 377 decarboxylase and the study of its role in regulating fatty acid metabolism. *Biochem J*
 378 350 Pt 2:599–608.

379 Dyck JRB, Hopkins TA, Bonnet S, Michelakis ED, Young ME, Watanabe M, Kawase Y,
 380 Jishage K, Lopaschuk GD (2006) Absence of malonyl coenzyme A decarboxylase in
 381 mice increases cardiac glucose oxidation and protects the heart from ischemic injury.
 382 *Circulation* 114:1721–8. doi: 10.1161/CIRCULATIONAHA.106.642009

383 Folmes CDL, Park S, Terzic A (2013) Lipid metabolism greases the stem cell engine. *Cell*
 384 *Metab* 17:153–5. doi: 10.1016/j.cmet.2013.01.010

385 Froese DS, Forouhar F, Tran TH, Vollmar M, Kim YS, Lew S, Neely H, Seetharaman J,
 386 Shen Y, Xiao R, Acton TB, Everett JK, Cannone G, Puranik S, Savitsky P, Krojer T,
 387 Pilka ES, Kiyani W, Lee WH, Marsden BD, von Delft F, Allerston CK, Spagnolo L,
 388 Gileadi O, Montelione GT, Oppermann U, Yue WW, Tong L (2013) Crystal structures
 389 of malonyl-coenzyme A decarboxylase provide insights into its catalytic mechanism and
 390 disease-causing mutations. *Structure* 21:1182–92. doi: 10.1016/j.str.2013.05.001

391 Haan EA, Scholem RD, Croll HB, Brown GK (1986) Malonyl coenzyme A decarboxylase
 392 deficiency. Clinical and biochemical findings in a second child with a more severe
 393 enzyme defect. *Eur J Pediatr* 144:567–70.

394 Hwang I-W, Makishima Y, Kato T, Park S, Terzic A, Park EY (2014) Human acetyl-CoA
 395 carboxylase 2 expressed in silkworm *Bombyx mori* exhibits posttranslational
 396 biotinylation and phosphorylation. *Appl Microbiol Biotechnol* 98:8201–9. doi:
 397 10.1007/s00253-014-5715-6

398 Ishikiriya M, Nishina T, Kato T, Ueda H, Park EY (2009) Human single-chain antibody
 399 expression in the hemolymph and fat body of silkworm larvae and pupae using BmNPV
 400 bacmids. *J Biosci Bioeng* 107:67–72. doi: 10.1016/j.jbiosc.2008.11.001

401 Kamionka M (2011) Engineering of therapeutic proteins production in *Escherichia coli*. *Curr*
 402 *Pharm Biotechnol* 12:268–74.

403 Karger AB, Park S, Reyes S, Bienengraeber M, Dyer RB, Terzic A, Alekseev AE (2008)
 404 Role for SUR2A ED domain in allosteric coupling within the K(ATP) channel complex.
 405 *J Gen Physiol* 131:185–96. doi: 10.1085/jgp.200709852

406 Kato T, Kajikawa M, Maenaka K, Park EY (2010) Silkworm expression system as a platform
 407 technology in life science. *Appl Microbiol Biotechnol* 85:459–70. doi: 10.1007/s00253-
 408 009-2267-2

409 Kim KH, López-Casillas F, Bai DH, Luo X, Pape ME (1989) Role of reversible
 410 phosphorylation of acetyl-CoA carboxylase in long-chain fatty acid synthesis. *FASEB J*
 411 3:2250–6.

412 Kim YS, Kolattukudy PE (1978) Purification and properties of malonyl-CoA decarboxylase
 413 from rat liver mitochondria and its immunological comparison with the enzymes from
 414 rat brain, heart, and mammary gland. Arch Biochem Biophys 190:234–46.

415 Kim YS, Kolattukudy PE, Boos A (1979) Malonyl-CoA decarboxylase in rat brain
 416 mitochondria. Int J Biochem 10:551–5.

417 Krawinkel MB, Oldigs HD, Santer R, Lehnert W, Wendel U, Schaub J (1994) Association of
 418 malonyl-CoA decarboxylase deficiency and heterozygote state for haemoglobin C
 419 disease. J Inherit Metab Dis 17:636–7.

420 Laurent G, German NJ, Saha AK, de Boer VCJ, Davies M, Koves TR, Dephoure N, Fischer
 421 F, Boanca G, Vaitheesvaran B, Lovitch SB, Sharpe AH, Kurland IJ, Steegborn C, Gygi
 422 SP, Muoio DM, Ruderman NB, Haigis MC (2013) SIRT4 coordinates the balance
 423 between lipid synthesis and catabolism by repressing malonyl CoA decarboxylase. Mol
 424 Cell 50:686–98. doi: 10.1016/j.molcel.2013.05.012

425 Lee GY, Bahk YY, Kim YS (2002) Rat malonyl-CoA decarboxylase; cloning, expression in
 426 *E. coli* and its biochemical characterization. J Biochem Mol Biol 35:213–9.

427 MacPhee GB, Logan RW, Mitchell JS, Howells DW, Tsotsis E, Thorburn DR (1993)
 428 Malonyl coenzyme A decarboxylase deficiency. Arch Dis Child 69:433–6.

429 Matalon R, Michaels K, Kaul R, Whitman V, Rodriguez-Novo J, Goodman S, Thorburn D
 430 (1993) Malonic aciduria and cardiomyopathy. J Inherit Metab Dis 16:571–3.

431 Motohashi T, Shimojima T, Fukagawa T, Maenaka K, Park EY (2005) Efficient large-scale
 432 protein production of larvae and pupae of silkworm by *Bombyx mori* nuclear

433 polyhedrosis virus bacmid system. *Biochem Biophys Res Commun* 326:564–9. doi:
 434 10.1016/j.bbrc.2004.11.060

435 Nam HW, Lee GY, Kim YS (2006) Mass spectrometric identification of K210 essential for
 436 rat malonyl-CoA decarboxylase catalysis. *J Proteome Res* 5:1398–406. doi:
 437 10.1021/pr050487g

438 Ogata M, Nakajima M, Kato T, Obara T, Yagi H, Kato K, Usui T, Park EY (2009) Synthesis
 439 of sialoglycopolypeptide for potentially blocking influenza virus infection using a rat
 440 alpha2,6-sialyltransferase expressed in BmNPV bacmid-injected silkworm larvae. *BMC*
 441 *Biotechnol* 9:54. doi: 10.1186/1472-6750-9-54

442 Otsuki T, Dong J, Kato T, Park EY (2013) Expression, purification and antigenicity of
 443 *Neospora caninum*-antigens using silkworm larvae targeting for subunit vaccines. *Vet*
 444 *Parasitol* 192:284–7. doi: 10.1016/j.vetpar.2012.09.038

445 Park EY, Abe T, Kato T (2008a) Improved expression of fusion protein using a cysteine-
 446 protease- and chitinase-deficient *Bombyx mori* (silkworm) multiple
 447 nucleopolyhedrovirus bacmid in silkworm larvae. *Biotechnol Appl Biochem* 49:135–
 448 140. doi: 10.1042/BA20070098

449 Park EY, Kageshima A, Kwon M-S, Kato T (2007) Enhanced production of secretory
 450 beta1,3-N-acetylglucosaminyltransferase 2 fusion protein into hemolymph of *Bombyx*
 451 *mori* larvae using recombinant BmNPV bacmid integrated signal sequence. *J Biotechnol*
 452 129:681–8. doi: 10.1016/j.jbiotec.2007.01.028

453 Park H, Kaushik VK, Constant S, Prentki M, Przybytkowski E, Ruderman NB, Saha AK
 454 (2002) Coordinate regulation of malonyl-CoA decarboxylase, sn-glycerol-3-phosphate

455 acyltransferase, and acetyl-CoA carboxylase by AMP-activated protein kinase in rat
 456 tissues in response to exercise. *J Biol Chem* 277:32571–7. doi:
 457 10.1074/jbc.M201692200

458 Park S, Hwang I-W, Makishima Y, Perales-Clemente E, Kato T, Niederländer NJ, Park EY,
 459 Terzic A (2013) Spot14/Mig12 heterocomplex sequesters polymerization and restrains
 460 catalytic function of human acetyl-CoA carboxylase 2. *J Mol Recognit* 26:679–88. doi:
 461 10.1002/jmr.2313

462 Park S, Lim BBC, Perez-Terzic C, Mer G, Terzic A (2008b) Interaction of asymmetric
 463 ABCC9-encoded nucleotide binding domains determines KATP channel SUR2A
 464 catalytic activity. *J Proteome Res* 7:1721–8. doi: 10.1021/pr7007847

465 Pender C, Trentadue AR, Pories WJ, Dohm GL, Houmard JA, Youngren JF (2006)
 466 Expression of genes regulating Malonyl-CoA in human skeletal muscle. *J Cell Biochem*
 467 99:860–867. doi: 10.1002/jcb.20944

468 Saggerson D (2008) Malonyl-CoA, a key signaling molecule in mammalian cells. *Annu Rev*
 469 *Nutr* 28:253–72. doi: 10.1146/annurev.nutr.28.061807.155434

470 Saha AK, Schwarsin AJ, Roduit R, Masse F, Kaushik V, Tornheim K, Prentki M, Ruderman
 471 NB (2000) Activation of malonyl-CoA decarboxylase in rat skeletal muscle by
 472 contraction and the AMP-activated protein kinase activator 5-aminoimidazole-4-
 473 carboxamide-1-beta -D-ribofuranoside. *J Biol Chem* 275:24279–83. doi:
 474 10.1074/jbc.C000291200

475 Sambandam N, Steinmetz M, Chu A, Altarejos JY, Dyck JRB, Lopaschuk GD (2004)
 476 Malonyl-CoA decarboxylase (MCD) is differentially regulated in subcellular

compartments by 5'AMP-activated protein kinase (AMPK). Studies using H9c2 cells overexpressing MCD and AMPK by adenoviral gene transfer technique. *Eur J Biochem* 271:2831–40. doi: 10.1111/j.1432-1033.2004.04218.x

Samokhvalov V, Ussher JR, Fillmore N, Armstrong IKG, Keung W, Moroz D, Lopaschuk DG, Seubert J, Lopaschuk GD (2012) Inhibition of malonyl-CoA decarboxylase reduces the inflammatory response associated with insulin resistance. *Am J Physiol Endocrinol Metab* 303:E1459–68. doi: 10.1152/ajpendo.00018.2012

Scholte HR (1969) The intracellular and intramitochondrial distribution of malonyl-CoA decarboxylase and propionyl-CoA carboxylase in rat liver. *Biochim Biophys Acta* 178:137–44.

Voilley N, Roduit R, Vicaretti R, Bonny C, Waeber G, Dyck JR, Lopaschuk GD, Prentki M (1999) Cloning and expression of rat pancreatic beta-cell malonyl-CoA decarboxylase. *Biochem J* 340 (Pt 1:213–7.

Xue J, Peng J, Zhou M, Zhong L, Yin F, Liang D, Wu L (2012) Novel compound heterozygous mutation of MLYCD in a Chinese patient with malonic aciduria. *Mol Genet Metab* 105:79–83. doi: 10.1016/j.ymgme.2011.09.007

Yano S, Sweetman L, Thorburn DR, Mofidi S, Williams JC (1997) A new case of malonyl coenzyme A decarboxylase deficiency presenting with cardiomyopathy. *Eur J Pediatr* 156:382–3.

Zhou D, Yuen P, Chu D, Thon V, McConnell S, Brown S, Tsang A, Pena M, Russell A, Cheng J-F, Nadzan AM, Barbosa MS, Dyck JRB, Lopaschuk GD, Yang G (2004)

498 Expression, purification, and characterization of human malonyl-CoA decarboxylase.

499 Protein Expr Purif 34:261–9. doi: 10.1016/j.pep.2003.11.023

500

Figure and Table Legends

Fig. 1 A schematic diagram of recombinant $\Delta 39\text{aa-hMCD}$ bacmid construction and expression in silkworm. The $\Delta 39\text{aa-hMCD}$ gene was amplified by PCR. This PCR fragment was digested by restriction enzymes and ligated into pFastbac1 vector. The pFastbac- $\Delta 39\text{aa-hMCD}$ was transformed into *E. coli* BmDH10Bac harboring cysteine protease- and chitinase-deficient *Bombyx mori* nucleopolyhedrovirus (*BmNPV-CP⁻-Chi⁻*). The constructed recombinant bacmid was directly injected into the dorsum of the larvae on the first day of their fifth-instar and pupae. M, mitochondrial-targeting sequence; K, Kozak sequence; F, FLAG tag; SKL, peroxisomal-targeting sequence. Black and green arrows denote bacmid constructions for $\Delta 39\text{aa-hMCD}$ and for $\Delta 39\text{aa-hMCD}$ mutants, respectively.

Fig. 2 SDS-PAGE (A) and Western blot (B) of recombinant $\Delta 39\text{aa-hMCD}$ expressed in silkworm fat body and pupae. MW, molecular weight markers; FB, $\Delta 39\text{aa-hMCD}$ from fat body; P, $\Delta 39\text{aa-hMCD}$ from pupae. Protein in homogenate was extracted with lysis buffer and purified by anti-FLAG M2 affinity gel column. Mock sample indicates bacmid, without hMCD gene, injected silkworm. An anti-FLAG M2 and an anti mouse IgG-HRP were used to detect $\Delta 39\text{aa-hMCD}$, and an anti-phosphoserine antibody was used to detect phosphorylation of $\Delta 39\text{aa-hMCD}$.

Fig. 3 Amino acid sequence alignments of human, rat and mouse MCD. (A). Blue, two initiating methionines; Red, putative phosphorylation site residues of hMCD by Netphos 2.0 prediction server; Arrow, point mutation sites for dephosphorylation of hMCD in this study. (B) Predicted phosphorylation sites of hMCD. Prediction score is the probability of phosphorylation ($\text{max} = 1$). (C) A point mutation in hMCD replaced serine (S) 204 (codon

TCA) with glycine (G) (codon GGA) and tyrosine (Y) 405 (codon TAC) with phenylalanine (F) (codon TTC).

Fig. 4 Western blot of purified $\Delta 39\text{aa-hMCD}$ and their mutants. MW, molecular weight markers; WT, wild type $\Delta 39\text{aa-hMCD}$; S204G, mutated $\Delta 39\text{aa-hMCD}$ at Serine 204; Y405F, mutated $\Delta 39\text{aa-hMCD}$ at Tyrosine 405; FB and P denote proteins purified from fat body and pupae, respectively. A monoclonal anti-phosphoserine and anti-phosphotyrosine antibodies produced in mouse were used as primary antibodies, and anti-mouse IgG-HRP was used as a secondary antibody. In western blot, 5 μg of proteins per well were used.

Fig. 5 Molecular organization of human peroxisomal MCD (PDB accession number: 2YGW). (A) A cartoon representation of MCD monomer composed of an N-terminal helical domain and a C-terminal catalytic domain where the catalytic acetyl-CoA binding site is located. (B) Structural arrangement of MCD tetramer. Inter-subunit disulfide bridges, Cys-206—Cys-206 in red spheres and Cys-243—Cys-243 in blue spheres, link the four subunits of tetramer. (C) MCD with phosphorylated residues of Ser-204 and Tyr-405. Cys-206 connects two subunits through the disulfide bond. (D) Tyr-405, located in the vicinity of the catalytic dyad of Ser-329 and His-423, forms a hydrogen bond with Asn-417. Cartoon MCD structures were generated with PyMol.

Table 1. The yields of recombinant proteins from silkworm larva or pupa

Table 2. Purification of $\Delta 39\text{aa-hMCD}$ from silkworm fat body and pupae

Table 3. Specific activities of purified $\Delta 39\text{aa-hMCD}$ mutants from silkworm fat body and pupae

Table 1. The yields of recombinant proteins from silkworm larva or pupa

Protein	μg/larva	μg/pupa	Reference
GFPuv-β3GnT2	91		Park et al. 2007
Anti-BSA scFv	188		Ishikiriya et al. 2009
ST6Gal1	220		Ogata et al. 2009
SAG1	170		Otsuki et al. 2013
SRS2	20		Otsuki et al. 2013
ACC2	150		Park et al. 2013
ACC2		500	Hwang et al. 2014
MCD	119	344	In this study

GFP, green fluorescent protein; β3GnT2, beta1,3-N-acetylglucosaminyl transferase 2; scFv, Human single-chain Fv fragment; ST6Gal1, rat alpha2,6-sialyltransferase; SAG1, *N. caninum* surface antigen 1; SRS2, SAG1-related sequence 2; ACC2, acetyl-CoA carboxylase 2; MCD, malonyl-CoA decarboxylase.

Table 2. Purification of $\Delta 39\text{aa-hMCD}$ from silkworm fat body and pupae

	Volume (mL/silkworm)	Protein (mg/silkworm)	Specific activity (nmol/mg/min)	Relative activity
Fat body				
Homogenate	3	193	19.61 ± 3.96	1
Purified $\Delta 39\text{aa-hMCD}$	0.5	0.119	59.54 ± 7.68	3
Pupae				
Homogenate	3	232	12.81 ± 3.36	1
Purified $\Delta 39\text{aa-hMCD}$	0.5	0.344	48.16 ± 7.89	3.7

Table 3. Specific activities of purified $\Delta 39\text{aa-hMCD}$ mutants from silkworm fat body and pupae

	Specific activity (nmol/mg/min)	Relative activity
Fat body		
$\Delta 39\text{aa-hMCD}$	59.54 ± 7.68	1
S204G	30.36 ± 2.25	0.51
Y405F	33.45 ± 3.56	0.56
Pupae		
$\Delta 39\text{aa-hMCD}$	48.16 ± 7.89	1
S204G	24.37 ± 1.99	0.51
Y405F	31.24 ± 1.69	0.64

550

551

(A)

HUMAN MRGFGPGLTARRLLPLRLPPRPPGPRLASGQAAGALERAMDELLRRVPPTPAYELREKT 60
 RAT MRGLGPSRLARRLLPLRYPPRPPGPRGR-LCSGLTASAMDELLRRVPPTPAYELREKT 59
 MOUSE MRGLGPGLTARRLLPLRSPRPPGPRGR-LCGGLAASAMDELLRRVPPTPAYELREKT 59
 :*. * ** *

HUMAN PAPAEGQCADFVSFYGGLAETAQRAELLGRLARGFGVDHGQVAEQSAGVLHLRQQQREAA 120
 RAT PAPAEGQCADFVSFYGGLAEEAQAELLGRLAQGFVDHGQVAEQSAGVLQLRQQSREAA 119
 MOUSE PAPAEGQCADFVSFYGGLAEASQRAELLGRLAQGFVDHGQVAEQSAGVLQLRQQAREAA 119
 *****:*****:*****:***** *

HUMAN VLLQAEDRLRYALVPRYRGLFHHISKLDGGVRFLVQLRADLLEAQALKLVEGPDVREMNG 180
 RAT VLLQAEDRLRYALVPRYRGLFHHISKLDGGVRFLVQLRADLLEAQALKLVEGPHVREMNG 179
 MOUSE VLLQAEDRLRYALVPRYRGLFHHISKLDGGVRFLVQLRADLLEAQALKLVEGPHVREMNG 179
 *****:***** *

↓

HUMAN VLKGM LSEWFSSGFLNLERVTWSPCEVLQKISEAEVHPVKNWMDMKRRVGPYRRCYFF 240
 RAT VLKSM LSEWFSSGFLNLERVTWSPCEVLQKISEAEVHPVKNWMDMKRRVGPYRRCYFF 239
 MOUSE VLKSM LSEWFSSGFLNLERVTWSPCEVLQKISEAEVHPVKNWMDMKRRVGPYRRCYFF 239
 . ** *

HUMAN SHCSTPGEPLVVLHVALTGDISSNIQAI VKEHPPSETEENKNI TAAIFYSISLTQQGLQG 300
 RAT SHCSTPGDPLVVLHVALTGDISNNIQSI VKECPPSETEENKNI AA AVFYSISLTQQGLQG 299
 MOUSE SHCSTPGEPLVVLHVALTGDISNNIQGI VKECPPTETEENKNI AAAIFYSISLTQQGLQG 299
 *****:*****:***. **** *:***:***:***** *

HUMAN VELGTFLIKRVVKELQREFPHLGVFSSLSPIPGFTKWLLGLLNSQTKEHGRNELFTDSEC 360
 RAT VELGTFLIKRVVKELQKEFPHLGAFSSLSPIPGFTKWLLGLLNVQGKEYGRNELFTDSEC 359
 MOUSE VELGTFLIKRVVKELQKEFPQLGAFSSLSPIPGFTKWLLGLLNVQGKEYGRNELFTDSEC 359
 *****:***:*. ***** *

↓

HUMAN KEISEITGGPINETLKLLSSSEWVQSEKLVRALQPLMRLCAWLYGEKHRGYALNPVA 420
 RAT KEIAEVTGDPVHESLKGLSSGEWAKSEKLAQALCGPLMRLCAWLYGEKHRGYALNPVA 419
 MOUSE QEISAVTGNPVHESLKGLSSGEWVKSEKLTQALCGPLMRLCAWLYGEKHRGYALNPVA 419
 : **:*. **:*. **:*. **:*. **:*. *** *

HUMAN NFHLQNGAVLWRI NWMADVSLRGITGSCGLMANYRYFLEETGPNSTSYLGSKNIKASEQV 480
 RAT NFHLQNGAVMWRI NWMADSSKGLTSSCGLMVNYRYYLEETGPNSISYLGSKNIKASEQI 479
 MOUSE NFHLQNGAVMWRI NWMADSSKGLTSSCGLMVNYRYYLEETGPNSISYLGSKNIKASEQI 479
 *****:***** **:*. **:*. **:*. **:*. ***** *

HUMAN LSLVAQFQKNSKL 493
 RAT LSLVAQFQNSKL 492
 MOUSE LSLVAQFQNSKL 492
 ***** *

(B)

Amino acid	Prediction Score
Ser204	0.980
Ser275	0.997
Ser329	0.905
Ser380	0.791
Thr9	0.893
Thr60	0.982
Thr245	0.869
Thr396	0.772
Tyr468	0.541

Applied Microbiology and Biotechnology

Supporting information

Phosphorylation of Ser-204 and Tyr-405 in human malonyl-CoA decarboxylase expressed in silkworm *Bombyx mori* regulates catalytic decarboxylase activity

In-Wook Hwang · Yu Makishima · Tomohiro Suzuki · Tatsuya Kato · Sungjo Park · Andre Terzic · Shin-kyo Chung · Enoch Y. Park

In-Wook Hwang · Enoch Y. Park (✉)

Laboratory of Biotechnology, Integrated Bioscience Section, Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka, 422-8529, Japan. E-mail: park.enoch@shizuoka.ac.jp; Tel. & Fax: +81-54-2384887

Yu Makishima · Tatsuya Kato · Enoch Y. Park

Laboratory of Biotechnology, Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka, 422-8529, Japan.

Tomohiro Suzuki

Research Institute of Green Science and Technology, Shizuoka University, 836 Ohya Suruga-ku, Shizuoka, 422-8529, Japan.

Tatsuya Kato · Enoch Y. Park

Laboratory of Biotechnology, Green Chemistry Research Division, Research Institute of Green Science and Technology, Shizuoka University, 836 Ohya Suruga-ku, Shizuoka, 422-8529, Japan.

Sungjo Park · Andre Terzic

Center for Regenerative Medicine, Mayo Clinic, 200 First Street SW, Rochester, MN, 55905, USA. Marriott Heart Disease Research Program, Division of Cardiovascular Diseases, Departments of Medicine, Molecular Pharmacology and Experimental Therapeutics, and Medical Genetics, Mayo Clinic, 200 First Street SW, Rochester, MN, 55905, USA.

Shin-kyo Chung

School of Food Science and Biotechnology, Kyungpook National University, Daegu 702-701, Republic of Korea

Authors' E-mail address;

In-Wook Hwang: gunryung21@hanmail.net (IH)

Yu Makishima: mmm.yu@live.jp (YM)

Tomohiro Suzuki: atsuzuk@ipc.shizuoka.ac.jp (TS)

Tatsuya Kato: atkato@ipc.shizuoka.ac.jp (TK)

Sungjo Park: park.sungjo@mayo.edu (SP)

Andre Terzic: terzic.andre@mayo.edu (AT)

Shin-kyo Chung: kchung@knu.ac.kr (SC)

Enoch Y. Park: park.enoch@shizuoka.ac.jp (EYP)

Figure S2. A proposed model of malonyl-CoA decarboxylase (MCD) phosphorylation in regulating lipid metabolism. Phosphorylation of Ser-204 and Tyr-405 in MCD enhances malonyl-CoA decarboxylation by reducing malonyl-CoA levels in cytoplasm, which promotes a stimulation of long chain acryl-CoA (LCACoA) oxidation by releasing the malonyl-CoA inhibition of carnitine palmitoyl transferase 1 (CPT1). In addition to MCD dependent regulation, phosphorylation of acetyl-CoA carboxylase (ACC) by AMP-activated kinase (AMPK) or inhibition of ACC by Spot14/Mig12 diminishes malonyl-CoA levels, subsequently promoting lipid oxidation.

Figure S3. Construction of recombinant bacmid by Bac-to-Bac system and protein production using silkworms. Amp^R; Ampicillin resistance, Gm^R; Gentamycin resistance, Km^R; kanamycin resistance, Tet^R; tetracycline resistance, P_{PH}; polyhedrin promoter.